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Exposure to specific neurotoxins can cause Parkinson's disease by damaging dopamnergic neurons. These neurotoxins induce neuronal loss by apoptosis or cell death, which results from sequential signaling events, from the inducer of apoptosis to the ultimate execution of the cellular changes characteristic of this process. We provide evidence that structurally distinct classes of neurotoxins activate the tumor suppressor protein, p53 early after toxic insult to induce cell death. The protection observed with inhibition of p53 further confirmed that p53 activation is critical for the cell loss in response to neurotoxins. Thus, the p53 cascade, or factors upstream regulating p53 activation, can be used as targets for neuroprotective strategies in models of Parkinson's disease. The drugs acting on dopamine receptors are known to provide symptomatic benefits for Parkinson's disease, recent clinical trials suggest that they might also be neuroprotective. We find that dopamine agonists have robust, concentration-dependent antiapoptotic activity. The predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-K and Akt. We also found that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in their coupling to G-proteins and to a cytoprotective c-Src/EGFR-mediated PI-3K/Akt pathway.

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Molecular Mechanisms of Dopamine Receptor Mediated Neuroprotection

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A. Introduction

Exposure to specific neurotoxins can cause symptoms of Parkinson's disease (PD) by damaging dopaminergic neurons. These neurotoxins can cause neuronal loss by inducing apoptosis or cell death, which results from sequential signaling events, from the inducer of cell death to the ultimate execution of the cellular changes characteristic of this process. Evidence from our laboratory and others suggest that agonists acting at dopamine (DA) receptors can oppose the induction of cell death in dopamine neurons. Moreover, recent clinical trials raise the possibility that they may have neuroprotective effects in PD as well.

The mechanisms underlying the induction of cell death by neurotoxins or the DA agonist-mediated neuroprotection are poorly understood. In this final report, we present evidence that distinct classes of neurotoxins such as the structural analogues of dopamine (6-hydroxydopamine (6-OHDA)), the mitochondrial complex I inhibitors (1-methyl-4-phenyl-pyridinium ion (MPP^+) and rotenone) and the proteasome inhibitors (MG132 and lactacystin) all induce an early signaling mechanisms in dopaminergic neurons. In this pathway, the tumor suppressor protein, p53 is phosphorylated at serine15 site, a pattern predicted to cause cell death in various experimental systems. All neurotoxins tested caused activation of p53 and loss of cell viability, however only 6-hydroxydopamine and 1-methyl-4-phenylpyridinium ion caused casapse-3 activation. Consistent with caspase-3 activation, in 6-OHDA treated cells the phosphorylated p53 was localized in the nucleus and induced p53-dependent PUMA gene expression. The translocation of phopshorylated p53 into cytoplasm in response to proteasome inhibitior, MG132 correlated with its inability to activate caspase-3 and PUMA induction. However, the protection observed with inhibition of p53 by pifithrin alpha against neurotoxins further confirmed that p53 activation is critical for the cell loss in response to neurotoxins. Our results suggest that p53 acts as a common mediator for divergent neurotoxins to induce cell death by a caspase-dependent or -independent pathway. Thus, the p53 cascade, or factors upstream regulating p53 activation, can be used as targets for neuroprotective strategies in models of Parkinson's disease. The results are summarized in appendix 1 [1] and 2.

The drugs acting on dopamine receptors are known to provide symptomatic benefits for PD, recent clinical trials suggest that they might also be neuroprotective. We find that DA agonists have robust, concentration-dependent antiapoptotic activity in dopaminergic cell line, PC12 that stably express human D_{2L} receptors from cell death

due to oxidative stress or trophic withdrawal and that the protective effects are abolished in the presence of D_2-receptor antagonists. D_2 agonists are also neuroprotective in the nigral dopamine cell line SN4741, which express endogenous D_2 receptors, whereas no anti-apoptotic activity is observed in native PC12 cells, which do not express detectable D_2 receptors. Notably, the agonists studied differ in their relative efficacy to mediate antiapoptotic effects and in their capacity to stimulate [^{35}S] guanosine 5 -[γ -thio] triphosphate ([^{35}S]GTP[γ S]) binding, an indicator of G protein activation. Studies with inhibitors of phosphoinositide 3-kinase (PI 3-K), extracellular-signal-regulated kinase or p38 mitogen-activated protein kinase indicate that the PI 3-K pathway is required for D_2 receptor-mediated cell survival. These studies indicate that certain DA agonists can complex with D_2 receptors to preferentially transactivate neuroprotective signaling pathways and to mediate increased cell survival. The results are summarized in appendix 3 [2].

We also report the characterization of the D_2 receptor signaling pathways mediating the cytoprotection. Bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either PI 3-K, epidermal growth factor receptor (EGFR), or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress. Co-immunoprecipitation studies showed that the D_2 receptor forms a complex with the EGFR and c-Src that was augmented by bromocriptine, suggesting a cross talk between these proteins in mediating the activation of Akt. EGFR repression by inhibitor or by RNA interference eliminated the activation of Akt by bromocriptine. D_2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphorylation specifically at tyrosine 845, a known substrate of Src kinase. Furthermore, Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation. Thus, the predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-K and Akt. We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D_2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in this experimental system. These results support the hypothesis that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in there coupling to G-proteins and to a cytoprotective c-Src/EGFR-mediated PI-3K/Akt pathway. The results are summarized in appendix 4 [3].

Our findings on early signaling mechanisms activated by neurotoxins and the neuroprotective signal transduction pathways activated by DA agonists are clinically and militarily relevant to therapies that might prevent cell loss induced by neurotoxins.

B. Body

Statements of Work

OBJECTIVE 1: Determine the molecular mechanism underlying the cell death induced by oxidative stress, trophic withdrawl and L-Dopa induced cell death in dopaminergic cell lines and neurons.

OBJECTIVE 2: Determine the locus at which DA receptor activation interferes with the concatenated events mediating neurotoxin-initiated apoptosis in cell line and primary culture model systems.

OBJECTIVE 3: Determine the sequence of events leading from activation of a specific DA receptor to modulation of apoptosis.

OBJECTIVE 4: Determine the molecular mechanism underlying the agonist-specific activation of PI 3-K by the D_2 receptor.

Background and Progress:

Parkinson's disease (PD) is characterized by preferential degeneration of dopamine (DA) neurons in the substantia nigra pars compacta. Inhibition of oxidative phosphorylation, excitotoxicity, and generation of reactive oxygen species are considered important mediators of neuronal death in PD [4]. Recent studies suggest that apoptosis may play a role in the loss of DA neurons in PD [5]. The major executioners of apoptosis, caspases, are activated in dopaminergic substantia nigra neurons from PD patients [6, 7]. Distinct classes of neurotoxins: structural analogues of dopamine (such as 6-OHDA), mitochondrial complex I inhibitors (such as MPP^+ and rotenone), and proteasome inhibitors (such as lactacystin and MG132) all induce loss of dopaminergic neurons in experimental models that closely resemble Parkinson's disease in humans. However, the molecular mechanisms mediating degeneration of midbrain DA neurons in PD are poorly understood. We have demonstrated that when cells are exposed to oxidative stress they activate early signaling mechanisms directed towards either apoptosis or survival [1] (Appendix 1). We also present data suggesting that diverse neurotoxins act through a common early signaling protein, the p53, to induce cell death in *in vitro* culture models of PD and inhibition of p53 activity significantly increased cell survival following neurotoxin treatment (Appendix 2).

Laboratory studies demonstrate that DA agonists can protect dopaminergic neurons in a variety of tissue culture and *in vivo* models of PD [8]. In the clinic, dopamine agonists have long been employed as an adjunct to levodopa therapy in advanced PD patients who experience motor complication [9]. Prospective double blind clinical trials have also demonstrated that dopamine agonists can provide symptomatic benefits for early PD patients with a reduced risk of motor complications compared with levodopa [10, 11]. Recent clinical trials have reported that, in comparison to levodopa, DA agonists delay the rate of decline in neuroimaging surrogate markers of nigrostriatal function [12]. These clinical trials raise the possibility that DA agonists may slow the rate of disease progression and are neuroprotective in PD. There is however uncertainty as to the mechanisms responsible for these effects and how they might be protective in PD. Proposed mechanisms include levodopa sparing, direct anti-oxidant effects, stimulation of auto-receptors, and inhibition of subthalamic nucleus-mediated excitotoxicity [13]. In addition, some *in vitro* and *in vivo* studies have noted that the protective effects of DA agonists were eliminated when they were co-administered with D_2-receptor antagonists, suggesting that D_2 receptor activation may contribute to the neuroprotective effects observed in these models [14]. However, the mechanisms underlying the agonist-mediated neuroprotection reported in experimental models are

poorly understood and the potential for DA agonists to alter the clinical course of this disease remains an area of controversy [15].

Many heptahelical receptors couple to multiple signal transduction pathways, including various heterotrimeric G-protein-second messenger pathways and growth-factor receptor-protein kinase cascades [16]. The signal for activation of the proximal mediators of signaling such as heterotrimeric G-proteins, receptor kinases or other protein partners, is an alteration in the receptor's conformation that occurs following complexing with agonist. Studies in several heptahelical receptors suggest that these proteins exist in multiple, functionally significant conformations that may differ in their relative activation of different signaling pathways [17-20]. Studies with several receptors, including the dopamine D_2 receptor, suggest that agonists acting at the same receptor select among different active receptor conformations and determine the relative levels of activation of downstream signaling pathways, a hypothesis called agonist-directed signal trafficking [21-24].

In order to clarify the contribution of the D_2 receptor to DA-agonist mediated neuroprotection and to investigate the underlying mechanisms, we studied the effects of dopamine agonists in a PC12 cell line model system in the presence and absence of dopamine D_2 receptors. In these experiments, PC12 cells were induced to undergo apoptosis by either oxidative stress or trophic factor withdrawal. We found that certain DA agonists, but not all, could induce a robust increase in cell survival via activation of the D_2 receptors. Furthermore our results implicate PI 3-K in receptor mediated cell survival and suggest a dissociation between neuroprotective signaling pathways and the G-protein activation classically associated with D_2 receptor signaling. To elucidate the mechanisms underlying agonist-specific modulation of cell survival, we have now investigated the anti-apoptotic signaling pathway activated by the D_2 receptor. We find that D_2 receptor-mediated protection against oxidative stress involves a novel c-Src-dependent transactivation of the EGF receptor that activates PI 3-K/Akt and that agonists differ in their capacity to activate this pathway. The detailed results are summarized in our publications and attached as appendix 3 and 4.

To determine the domain(s) involved in PI 3-K activation by D_2R, we introduced mutations in wild type human D_{2L} receptor at potential functional domains present in the receptor (Table 1). Mutations were carried out using QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. For each point mutation, 1-3 substitutions were performed. The D_{2L} receptor mutants were characterized for their coupling to Gi G-protein by GTPγS assays when transfected into PC12 cells and for their capacity to mediate PH-Akt-GFP chimera translocation when co-transfected into PC12 cells. The detailed protocols were described in our publications [2, 3]. We were unable to identify D2 receptor functional domains involved in PI 3-K coupling after our extensive studies with mutants we have generated. Based on our results on the interaction between D_2 receptor and PI 3-K kinase [3], we will develop mutants that might elucidate additional function domains in D_2 receptor.

Table 1. List of D_2 receptor mutants studied

No	Position	Original amino acid	Substituted amino acid
1	141	Leu	Ala
2	141	Leu	Ser
3	142	Tyr	Ala
4	145	Tyr	Phe
5	145	Tyr	His
6	145	Tyr	Ser
7	150	Pro	Ala
8	150	Pro	Leu
9	213	Tyr	Ala
10	295	Tyr	Ala
11	325	Pro	Ala
12	358	Ser	Ala
13	358	Ser	Cys
14	370	Lys	Ala
15	370	Lys	Ser
16	371	Ala	Ser
17	436	Tyr	Phe
18	436	Tyr	Phe
19	438	Leu	Ala
20	438	Leu	Ser

C. Key Research Accomplishments

- Oxidative stress induces apoptosis in PC12 cells in a concentration and time dependent manner.
- In response to oxidative stress, PC12 cells activate signaling pathways of both homeostasis, as represented by activation of exrtracellular-regulated kinase (ERK) and pro-apoptotic responses as indicated by p53 activation.
- Individual cells segregate into two populations within the first hour of stress, either showing the gene induction mediated by activation of ERK or pre-apoptotic p53 activation.
- Changing the level of oxidative stress alters the relative proportion of pro-apoptotic cells at this early time point.
- Structurally distinct classes of neurotoxins activate p53 to induce cell death in dopaminergic cells.
- All neurotoxins tested caused activation of p53 and loss of cell viability, however only 6-OHDA and MPP^+ caused casapse-3 activation.
- Consistent with caspase-3 activation, in 6-hydroxydopamine treated cells the phosphorylated p53 was localized in the nucleus and induced p53-dependent PUMA gene expression.
- The translocation of phopshorylated p53 into cytoplasm in response to proteasome inhibitior, MG132 correlated with its inability to activate caspase-3 and PUMA induction.

- p53 inhibition by p53 inhibitor or D_2 agonist protects cells from the toxic effects of neurotoxins.
- The p53 cascade, or factors upstream regulating p53 activation, can be used as targets for neuroprotective strategies in models of PD.
- DA agonists mediate neuroprotection via activation of D_2 receptor against oxidative stress induced cell death in PC12 cells and immortalized nigral dopamine cells.
- D_2 receptor activates PI 3-K to mediate the neuroprotective effect of DA agonists.
- DA agonists differ in the relative efficacy to activate the classical G protein pathway and the neuroprotective PI 3-K pathway.
- We found large variations in the capacity of different D_2 agonists in mediating neuroprotection.
- The correlation of the efficacy of a particular agonist for treating the motor symptoms in PD and its neuroprotective activity in the *in vitro* assay we have developed is poor. Some symptomatically effective D_2 agonists appear to have low neuroprotective potential.
- Bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either PI 3-K, EGFR or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress.
- Large number of D_2 receptor mutants were constructed and characterized.
- Co-immunoprecipitation studies showed that activation of the D_2 receptor induced its association with the EGFR, suggesting a cross talk between these receptors in mediating the activation of Akt.
- EGFR repression by inhibitor or by RNA interference eliminated the activation of Akt by bromocriptine. D_2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphoryation specifically at tyrosine 845, a known substrate of Src kinase.
- Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation.
- The predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-kinase and Akt.
- We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D_2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in this experimental system.
- Our results support the hypothesis that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in their coupling to G proteins and to a cytoprotective EGFR-mediated PI-3 kinase/Akt pathway.

D. Reportable outcome

Manuscripts:

1. Nair, V. D., Yuen, T., Olanow, C. W. & Sealfon, S. C. Early single cell bifurcation of pro- and anti-apoptotic states during oxidative stress. *J. Biol. Chem.* **279**: 27494-27501. (Appendix 1)
2. Nair, V. D., Olanow, C. W. & Sealfon, S. C. Central role of p53 in divergent neurotoxin responses (manuscript to be submitted). (Appendix 4).
3. Nair, V. D., Olanow, C. W. & Sealfon, S. C. Activation of phosphoinositide 3-kinase by D_2 receptor prevents apoptosis in dopaminergic cell lines. *Biochem. J.* **373**: 25-32 (2003). (Appendix 3)
4. Nair, V. D. & Sealfon, S. C. Agonist specific transactivation of phosphoinositide 3-kinase signaling pathway mediated by the dopamine D_2 receptor. *J. Biol. Chem.* **278**: 47053-47061. (Appendix 4)

Abstracts:

1. Nair, V. D, Nelson, A. E & Sealfon, S. C. (2001) Oxidative stress induced early gene program in PC12 cells. Society for Neuroscience.
2. Nair, V. D. & Sealfon, S. C. (2002) Differential coupling of dopamine D_2 receptor to phosphoinositide 3-kinase mediates anti-apoptosis in PC12 cells. Society for Neuroscience.
3. Yuen, T., Nair, V. D. & Sealfon, S. C (2002). Coordinated apoptosis early gene program induced by oxidative stress in PC12 cells. Society for Neuroscience.

Development of cell lines:

Stable PC12 cell line expressing human dopamine D_2 receptor (PC12-D_2R).

Funding applied for based on work supported by this award:

Therapeutic control of the life/death switch in dopamine neurons
PI – Stuart C. Sealfon
National Institute of Health

Studies on D_2 agonists mediated neuroprotection in dopaminergic neurons.
PI – Venugopalan D. Nair
Funding Agency – Bachmann Strauss Dystonia and Parkinson's Foundation Inc.

Studies on the role of egr1 in cell survival
PI – Venugopalan Nair
Funding Agency - Bachmann Straus Parkinson and Dystonia Foundation Inc.

Oxidaitve stress activated RNA regulations
PI – Venugopalan Nair
National Institute of Health

Employment supported by this award:

Stuart C. Sealfon – Professor
Venugopalan D. Nair – Assistant Professor
Ashley nelson - Research Coordinator
Karen Said - Research Coordinator
Sejal Mehta - Research Coordinator

D. Conclusions

We have achieved the objectives of the proposed research and the progress during the funding period has been excellent. In this final report, we have incorporated all the changes recommended by the reviewers. The results promise to establish the foundation for the identification and implementation of neuroprotective therapies in diseases that are caused by toxin-induced cell death.

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Early Single Cell Bifurcation of Pro- and Antiapoptotic States during Oxidative Stress*[S]

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In a population of cells undergoing oxidative stress, an individual cell either succumbs to apoptotic cell death or maintains homeostasis and survives. Exposure of PC-12-D_2R cells to 200 μM hydrogen peroxide (H_2O_2) induces apoptosis in about half of cells after 24 h. After 1-h exposure to 200 μM H_2O_2, both antiapoptotic extracellular regulated kinase (ERK) phosphorylation and pro-apoptotic Ser-15-p53 phosphorylation are observed. Microarray and real-time PCR assays of gene expression after H_2O_2 exposure identified several transcripts, including *egr1*, that are rapidly induced downstream of ERK. Single cell analysis of *egr1* induction and of phospho-ERK and phospho-p53 formation revealed the presence of two distinct cellular programs. Whereas the proportion of cells activating ERK *versus* p53 at 1 h depended on H_2O_2 concentration, individual cells showed exclusively either phospho-p53 formation or activation of ERK and *egr1* induction. Exposure to H_2O_2 for 1 h also elicited these two non-overlapping cellular responses in both dopaminergic SN4741 cells and differentiated postmitotic PC-12-D_2R cells. Repressing p53 with pifithrin-α or small interfering RNA increased ERK phosphorylation by H_2O_2, indicating that p53-dependent suppression of ERK activity may contribute to the bi-stable single cell responses observed. By 24 h, the subset of cells in which ERK activity was suppressed exhibit caspase 3 activation and the nuclear condensation characteristic of apoptosis. These studies suggest that the individual cell rapidly and stochastically processes the oxidative stress stimulus, leading to an all-or-none cytoprotective or pro-apoptotic signaling response.

Reactive oxygen species (ROS)[1] have been implicated in the pathophysiology of several human diseases, including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative disorders, and aging (1–3). Although the cytotoxic actions of ROS are well known, ROS are increasingly recognized as components of cellular signaling that modulate responses in both physiological and pathological conditions (4). For example, ROS are produced in muscle cells upon binding of ligands such as angiotensin II (5). In addition, ROS production has been documented in a number of cells stimulated with cytokines, including tumor necrosis factor-α, transforming growth factor-β, and interleukin-1 (6–8), and with such growth factors as bovine fibroblast growth factor, nerve growth factor, platelet-derived growth factor, and epidermal growth factor (9–11). These observations suggest that ROS can damage various cell components or activate specific physiological signaling pathways, with the relative effects determined by ROS concentration.

Oxidative stress has been reported to activate seemingly contradictory signaling pathways, and the consequences of the response vary widely; the ultimate outcome is dependent on the balance between these stress-activated pathways (12, 13). Among the main stress signaling pathways and/or central mediators activated in response to oxidant injury are the extracellular regulated kinase (ERK) (14–16), c-*jun* amino-terminal kinase (JNK) (17–19), p38 mitogen-activated protein kinase (20) signaling cascades, the phosphoinositide 3-kinase/Akt pathway (21), the nuclear factor-κB signaling system (22, 23), p53 activation (24, 25), and the heat shock response (26). In general, the heat shock response, ERK, phosphoinositide 3-kinase/Akt, and nuclear factor-κB signaling pathways exert a pro-survival influence during oxidant injury, whereas activation of p53, JNK, and p38 are implicated in apoptosis (see review in Ref. 12).

ROS, including hydrogen peroxide (H_2O_2), are natural by-products generated by living organisms as a consequence of aerobic metabolism (27). The cellular toxicity of H_2O_2 is associated with the rapid modification of cellular constituents, including the depletion of intracellular glutathione and ATP, a decrease in NAD^+ level, an increase in free cytosolic Ca^{2+}, and lipid peroxidation (28). H_2O_2 also activates the opening of the mitochondrial permeability transition pore and the release of cytochrome *c* (29). In the cytoplasm, cytochrome *c*, in combination with Apaf-1, activates caspase-9 leading to the activation of caspase-3 and subsequent apoptosis (30). The initiating events leading to activation of these different signaling pathways in response to H_2O_2 are incompletely understood.

We have reported recently that H_2O_2 induces apoptosis in PC-12-D_2R cells and in the nigral dopaminergic neuronal cell line SN4741 in a concentration-dependent manner (31, 32). These observations suggest that when exposed to a level of oxidative stress that can induce apoptosis in a portion of cells, each individual cell must proceed through a decision-making process that ultimately results either in its survival or its death. We report here that early after H_2O_2 exposure, each cell activates either homeostatic or proapoptotic signaling pathways, but not both. Our results indicate that it may be chal-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

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[1] The abbreviations used are: ROS, reactive oxygen species; ERK, extracellular regulated kinase; JNK, c-*jun* N-terminal kinase; qPCR, quantitative real time PCR; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FISH, fluorescent *in situ* hybridization; siRNA, small interfering RNA.

lenging to develop models of the mechanism of oxidative stress-induced cell death based solely on cell population and biochemical assays.

EXPERIMENTAL PROCEDURES

Materials—U34 oligonucleotide array gene chips were from Affymetrix (Santa Clara, CA) and total RNA isolation kit was from Stratagene (La Jolla, CA). Antibodies specific to phospho-ERK, ERK, phospho-p53, p53, phospho-JNK, JNK, phospho-p38 kinase, and p38 kinase were from Cell Signaling Technology (Beverly, MA). Anti-active caspase-3 antibody was from Promega (Madison, WI). Enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from Amersham Biosciences (Piscataway, NJ). Alexa fluor 488, goat anti-mouse alexa fluor 568, and goat anti-rabbit alexa fluor 488 conjugated secondary antibodies were from Molecular Probes (Eugene, OR). CY3 and atlas nucleospin columns were from BD Biosciences Clontech. Donkey anti-rabbit CY3 was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Maxiscript *in vitro* transcription kit and aminoallyl-UTP were from Ambion (Austin, TX). PD98059 and was from Calbiochem (San Diego, CA), and pifithrin-α was obtained from A.G. Scientific (La Jolla, CA). Qiaex II gel extraction kit and pDrive vector were from Qiagen (Valencia, CA). All PCR reagents were from Invitrogen.

Cell Culture—PC-12-D_2R (31, 32) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 500 μg/ml G418 (Invitrogen), 10% horse and 5% fetal bovine serum (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37 °C. For differentiation, PC-12-D_2R cells were plated on to collagen-coated plates in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum and 100 ng/ml nerve growth factor for 7 days. Substantia nigra dopaminergic neuronal cell line SN4741was cultured as described previously (33).

Immunoblotting—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal C630, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, 5 μg/ml aprotinin, and mixture of protease inhibitors (Roche Diagnostics GmbH) at 4 °C for 20 min. After centrifugation at 14,000 × *g* for 20 min at 4 °C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and detected by immunoblotting using the ECL system according to the manufacturer's recommendations. The blots were then stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and re-probed with respective antibodies.

Gene Expression Analysis—PC-12-D_2R cells were treated with 200 μM H_2O_2 or vehicle for 1 h, and total RNA was isolated using StrataPrep total RNA miniprep kit according to the manufacturer's protocol. Preparation of cRNA, hybridization, and scanning of the rat genome U34 arrays were performed as described previously (34, 35). Affymetrix microarray suite 5.0 was used to analyze the raw data using the criteria of 60% concordance across multiple array comparisons and -fold changes ≥1.6 for outlier detection. For each of the up-regulated targets, quantitative real-time polymerase chain reaction (qPCR) was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using SYBR-Green assay, as described previously (35, 36). All the gene-specific primer sets used for qPCR are listed in supplemental table. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The comparative cycle threshold (C_T) method was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantitation for any given gene, expressed as -fold variation over control (untreated cells), was calculated after determination of the difference between C_T of the given gene A and that of the calibrator gene B (β-*actin*) in treated cells ($\Delta C_{T1} = C_{T1A} - C_{TB}$) and untreated cells ($\Delta C_{T0} = C_{T0A} - C_{TB}$) using the $2^{-\Delta\Delta CT\,(1-0)}$ formula (37). C_T values are means of triplicate measurements. Experiments were repeated three to five times.

Immunocytochemistry—PC-12-D_2R cells growing on collagen-coated or SN4741 cells growing on ploy-ornithine-coated cover glass were treated as indicated. The cells were fixed and permeabilized as described previously (31, 32), and immunocytochemical staining for phospho-ERK, phospho-p53, or active caspase-3 was carried out. Anti-phospho-ERK (1:400), anti-phospho-p53 (1:500), or active caspase-3 antibody (1:200) was added and incubated overnight at 4 °C. For double-immunolabeling, a mouse monoclonal anti-phospho-ERK antibody was used. After washing, cells were incubated with corresponding secondary antibodies for 2 h at room temperature. The cells were washed three times in phosphate-buffered saline, and the nuclei were stained with 1 μg/ml (in phosphate-buffered saline) of the fluorescent DNA dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min and then washed with phosphate-buffered saline. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium.

Fluorescent in Situ Hybridization—Egr1 (161 bp) and β-*actin* (150 bp) DNA fragments were amplified from RNA isolated from PC-12-D_2R cells by reverse transcription PCR using primer sets described in supplemental table. The DNA fragments were purified from agarose gel using QIAEX II gel extraction kit and sub-cloned into pDrive vector. Individual clones were sequenced to determine the orientation of the DNA. To generate cRNA probes, we used T7 promoter for antisense and SP6 promoter for sense probes to minimize the interference of vector sequences in double fluorescent *in situ* hybridization (FISH). Aminoallyl-UTP incorporated cRNA probes were generated using Maxiscript *in vitro* transcription kit. The yield and integrity of riboprobes was confirmed by gel electrophoresis. The *egr1* and β-*actin* cRNA probes were labeled with Alexa Fluor 488 and CY3, respectively, according to manufacturer's protocols. The riboprobes were purified on atlas nucleospin columns. The cells grown on cover slips were fixed and permeabilized as described previously (31, 32). After prehybridization in 5× SSC, 50% formamide, and 1 mg/ml tRNA at room temperature for 30 min, the denatured probe was added to the prehybridization buffer. Hybridization was carried out for 2 h at 52 °C. After two 5-min washes in 5× SSC, 50% formamide, 0.1% SDS, and twice in 2× SSC, the nuclei were stained with DAPI, and slides were mounted in Vectashield mounting medium. When immunostaining was carried out after FISH, the slides were incubated with respective antibodies as described above.

RNA Interference—Custom SMARTpool plus small interfering RNA (siRNA) to target rat p53 (GenBank™ accession number NM_030989) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (50 pmol) was transfected into PC-12-D_2R cells using transit-TKO transfection reagent (Mirus, Madison, WI) as described previously (32). After 48 h of transfection, cells were treated with H_2O_2 (200 μM) or vehicle for 1 h, and total RNA or cell extract was prepared. A nonspecific RNA duplex (Dharmacon) was used in control experiments.

RESULTS

ERK and p53 Pathways Are Activated in Response to Oxidative Stress—PC-12-D_2R cells and substantia nigra dopaminergic SN4741 cells undergo apoptosis when exposed to H_2O_2 in a concentration- and time-dependent manner (31, 38). Oxidative stress is known to activate multiple signal transduction pathways in many experimental systems (12). To identify the signaling mechanisms activated by PC-12-D_2R cells in response to H_2O_2, we have assessed the activation of ERK, JNK, p38-kinase, and p53, using Western blot analysis with antisera against phospho-ERK, phospho-JNK, phospho-p38 kinase, and phospho-p53. We found that 200 μM H_2O_2 rapidly induced the phosphorylation of ERK but not of JNK or p38 kinases in PC-12-D_2R cells (Fig. 1*A*, *B*, and *C*). The activation of ERK by H_2O_2 was rapid and sustained (Fig. 1*A*, *top*). Anti-phospho-Ser15 antibody was used to detect oxidative stress-induced phosphorylation of p53, presumably caused by DNA damage (39–41) after exposure to H_2O_2 for periods up to 6 h. In PC-12-D_2R cells, p53 phosphorylation was significantly enhanced within 30 min after H_2O_2 exposure and continued to increase for up to 2 h (Fig. 1*D*). The level of total p53 protein on Western blot analysis was unchanged after 6 h of incubation with H_2O_2 (Fig. 1*D*). These results demonstrate the early activation of ERK and p53 signaling pathways in response to oxidative stress in PC-12-D_2R cells.

Characterization of ERK-activated Gene Program in Response to Oxidative Stress—ROS, through its effects on cell signaling, alters the expression of specific genes (42). To identify the genomic response during oxidative stress, the gene expression profile associated with H_2O_2 exposure was studied using oligonucleotide microarrays and regulated transcripts were confirmed by qPCR. As shown in Table I, genes that encode transcription factors including *egr1*, c-*fos*, c-*jun*, *pc3*,

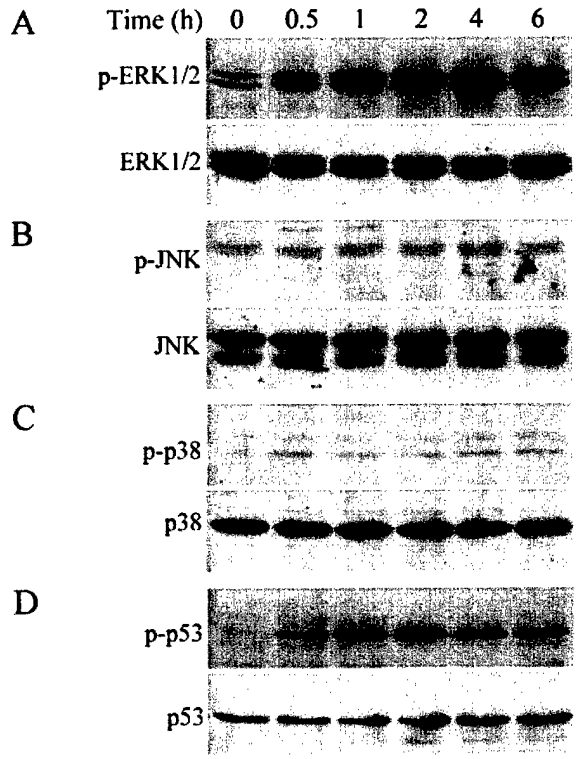

FIG. 1. **Overall response of signal transduction pathways to oxidative stress.** *A*, oxidative stress significantly increased ERK phosphorylation within 30 min after the addition of H_2O_2. *B* and *C*, H_2O_2 showed no effect on the phosphorylation of JNK and p38 kinase, respectively. *D*, H_2O_2 significantly increased p53 phosphorylation within 30 min after the addition of H_2O_2. The PC-12-D_2R cells were incubated with 200 μM H_2O_2 for the periods indicated, and immunoblots were carried out using specific phosphoprotein antibodies. The blots were stripped and reprobed using the antibodies recognizing total ERK, JNK, p38 kinase, or p53 proteins. The blots shown are representative of four independent experiments with similar results.

and a zinc finger protein (*copeb*) were up-regulated after 200 μM H_2O_2 for 1 h. Other up-regulated genes include inner mitochondrial membrane component ATP synthase subunit c, stress response gene 70-kDa heat shock protein (hsp70), and the immediate-early inducible small GTP binding protein rhoB. We also found that the mitogen-activated protein kinase phosphatase-1 (*mkp1*) was increased by >3-fold in cells treated with H_2O_2. Several immediate early genes, such as *egr1*, *c-fos*, and *c-jun*, have been reported to be transcriptionally activated by increased cellular oxidation (16, 43, 44). Oxidative stress has also been shown to induce rhoB (45) and hsp70 (26).

To identify the component of the oxidative stress-induced gene program downstream of ERK activation, we inhibited ERK with PD98059. Addition of PD98059 (100 μM) 1 h before H_2O_2 treatment decreased the induction of *egr1*, *pc3*, and *mkp1* (Fig. 2), genes that have been identified as downstream of ERK in other experimental systems (46–48). PD98059 did not prevent the induction of *c-fos*, *copeb*, *c-jun*, *hsp70*, *rhoB*, ATP synthase subunit c, and expressed sequence tag (GenBank™ accession number AI639167).

Characterization of egr1 Induction in Response to Oxidative Stress—To investigate the cellular segregation of the diverse responses to oxidative stress, we next studied the induction of *egr1* mRNA using FISH. We exposed cells to 200 μM H_2O_2, a concentration that induces apoptosis in approximately half of the cells (31). When cells were exposed to 200 μM H_2O_2, we observed that approximately half of the cells had strong fluorescent signals (Fig. 3). The signal in the subset of cells not showing *egr1* induction at this concentration of H_2O_2 was indistinguishable from that of control cells. Cells hybridized with sense-oriented probe for *egr1* showed no fluorescent signal in any cells (data not shown). Simultaneous double-FISH for egr1 and β-actin mRNA showed no change in β-actin mRNA expression during H_2O_2 treatment (Fig. 3). These data demonstrate that H_2O_2 induces a high level of *egr1* induction in a subset of cells and no detectable change in *egr1* levels in others.

ERK-mediated egr1 Induction and p53 Activation Were Present in Discrete Subsets of Cellular Populations—In many experimental systems, signaling through ERK is known to be prosurvival (16, 30, 49, 50) and p53 activation is known to be proapoptotic (25, 51–53). The p53 protein plays a central role in the cellular response to DNA damage that leads to phosphorylation and activation of p53 (53–55). To characterize the cellular segregation of signaling pathways simultaneously activated by ROS, we monitored the phosphorylation of ERK and p53 signaling pathways after H_2O_2 exposure using immunocytochemical staining of PC-12-D_2R cells. In response to H_2O_2, enhanced ERK phosphorylation was detected both in the cytoplasm and in the nucleus (Fig. 4*A*, *top*). However, phospho-p53 was mainly localized in the nucleus (Fig. 4*A*, *bottom*). Approximately half of the cells showed ERK or p53 phosphorylation in response to 200 μM H_2O_2 (Fig. 4*B*).

We next used double labeling to study whether there was overlap of p53 and pERK/*egr1* induction within the same cells. As shown in Fig. 5*A*, egr1 mRNA co-localized to cells showing ERK activation. However, the induction of egr1 mRNA by H_2O_2 was absent in cells showing p53 activation (Fig. 5*B*). These results indicate that oxidative stress activates ERK or p53 signaling pathways in separate cell subpopulations.

Relative Proportion of Cells Showing Either Activation of ERK or p53 Is Dependent on the Concentration of H_2O_2 in PC-12-D_2R and SN4741 Cells—We have demonstrated previously that PC-12-D_2R cells undergo concentration dependent apoptosis when exposed to H_2O_2 (31). In the present study, we have demonstrated that in undifferentiated PC-12-D_2R cells, 200 μM H_2O_2 activates ERK or p53, and the cells are segregated into separate cell subpopulations. To elucidate the role of these pathways in cell survival or death, we examined the effect of varying concentrations of H_2O_2 on ERK and p53 activation. As shown in Fig. 6*A*, low sublethal concentrations of H_2O_2 activated ERK but not p53. At higher concentrations of H_2O_2 (200–400 μM), phosphorylation of both ERK and p53 is observed. Consistent with our previous observation that the sublethal concentrations of H_2O_2 had no significant effect on the cell death (31), the activation of ERK and the absence of p53 phosphorylation by these concentrations of H_2O_2 suggest that ERK activation is antiapoptotic. At the single cell level, 100 μM H_2O_2 phosphorylated ERK and induced *egr1* in almost all the cells. However, 200 μM H_2O_2 activated ERK/*egr1* in only half of the cells (Fig. 6*B*).

To explore whether the ROS-mediated segregation of signaling pathways in separate cell subpopulations observed in PC-12-D_2R cells was present in a different cellular context, we studied this signaling pathways in a mouse immortalized nigral dopaminergic cell line SN4741 (33). Incubation of these cells with H_2O_2 was found to induce cell death in a concentration-dependent manner (31, 38). SN4741 cells undergo cell death at low concentrations of H_2O_2 (50–100 μM) compared with PC-12-D_2R cells. We found that H_2O_2 induced phosphorylation of both ERK and p53 (data not shown). At the single cell level, 50 μM H_2O_2 phosphorylated ERK and p53 in separate populations of cells (Fig. 7*B*). However, 100 μM H_2O_2 activated p53 in almost all the cells (Fig. 7*C*). These results suggest that ROS activates opposing signaling pathways in discrete cell sub-populations of dopaminergic neuronal cell line.

TABLE I

Effect of H_2O_2 on gene expression in PC-12 cells

Total RNA was isolated from PC-12-D_2R cells untreated or treated with 200 μM H_2O_2 for 1 h. Changes in the expression level of H_2O_2-induced genes in Affymetrix analysis ($n = 4$) were independently confirmed using qPCR analysis of RNA from control and H_2O_2-treated cells. The qPCR results are presented as -fold increase over control values using the 2 C_T formula as described under "Experimental Procedures." Data presented are mean ± S.E. of one experiment repeated three times with essentially the same results.

Identifier	Gene description	Fold change	
		Microarray	qPCR
AF023087	Nerve growth factor induced factor A (*egr1*)	5.66 ± 1.73	25.01 ± 1.12
M18416	*egr1*	4.25 ± 2.18	
U75397	*egr1*	3.78 ± 6.23	
S81478	Oxidative stress-inducible protein tyrosine phosphatase (*mkp1*)	4.12 ± 5.95	5.47 ± 1.12
AA945867	*c-jun*	4.12 ± 3.20	1.93 ± 0.30
AI175959	*c-jun*	2.58 ± 0.58	
M60921	NGF-inducible anti-proliferative putative secreted protein (*pc3*)	3.32 ± 3.70	5.50 ± 0.70
M60921	*pc3*	1.63 ± 0.26	
AA944156	*pc3*	1.58 ± 0.40	
AF001417	Core promoter element binding protein (*copeb*)	2.76 ± 1.99	2.18 ± 0.50
AI639167	Expressed sequence tag	2.33 ± 1.41	1.76 ± 0.42
D13123	P1 mRNA for ATP synthase subunit c	1.97 ± 0.54	1.70 ± 0.50
X06769	*c-fos*	1.92 ± 0.41	2.00 ± 0.70
AA900505	*RhoB*	1.83 ± 0.19	1.78 ± 0.60
L16764	Heat shock protein 70 (*hsp70*)	1.60 ± 0.41	1.45 ± 0.50

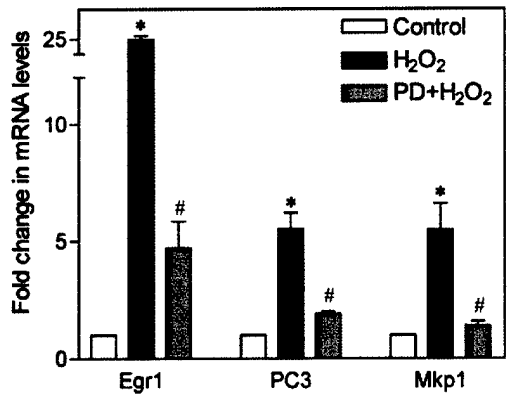

FIG. 2. **Effect of ERK inhibition on oxidative stress activated gene program.** Total RNA was isolated from control PC-12-D_2R cells or cells treated with 200 μM H_2O_2 for 1 h in the presence or absence of 100 μM PD980059 ($n = 5$). The changes in mRNA expression were measured using qPCR analysis. Results are presented as -fold increase over control values using the 2 C_T formula. Data presented are mean ± S.E. of one experiment repeated three times with essentially the same results. Significant variation between two groups was determined using Student's *t* test using Prism software, v. 3.0 (GraphPad Software Inc., San Diego, CA). *, $p < 0.001$ compared with control. #, $p < 0.001$ compared with H_2O_2.

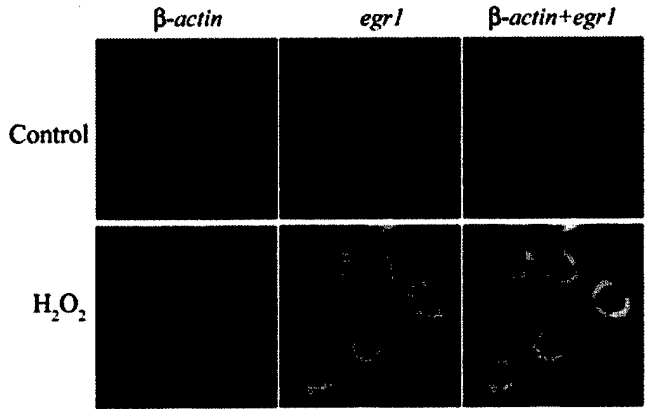

FIG. 3. ***Egr1* mRNA induction in response to H_2O_2.** Double-label FISH study for egr1 mRNA (*green*) and β-actin mRNA (*red*) of PC-12-D_2R cells exposed to vehicle or 200 μM H_2O_2 for 1 h. The nuclei were counterstained with DAPI (*blue*). Note that egr1 is strongly induced in a subpopulation of cells. The field shown is representative, and these results were replicated in four independent experiments.

Activation of ERK and p53 in Postmitotic PC-12-D_2R Cells in Response to H_2O_2—We have previously shown that 200 μM H_2O_2 induce cell death in proliferating and postmitotic PC-12-D_2R cells (31). In response to H_2O_2, proliferating cells activate ERK or p53 in separate cell subpopulations. One possible explanation for the opposite responses of individual cells to H_2O_2 could be that cells at different points in the cell cycle might differ in their response. An alternative explanation for our results would be that the initial state differences between cells showing opposite responses are relatively insignificant and the process is stochastic. To determine the influence of cell cycle on the early bifurcation of signaling mechanisms activated by ROS, we differentiated PC-12-D_2R cells for 7 days and analyzed the activation of ERK and p53 at the single cell level. As shown in Fig. 8, H_2O_2 (200 μM) activated ERK and p53 in separate populations of cells. These results are similar to those obtained in proliferating PC-12-D_2R cells. These results suggest that differences between cells that activate ERK or p53 are independent of cell cycle.

Caspase-3 Activation Occurs Only in Cells Not Showing ERK Activation in Response to Oxidative Stress—Double immunostaining for phospho-ERK and phospho-p53 showed that p53 and ERK are activated in different cells in the presence of 200 μM H_2O_2 for 1 h (Fig. 9*A*). In an attempt to elucidate the fate of cells showing either ERK or p53 activation, we examined activation of caspase-3, a mediator of cell death (31). When examined after 24 h of H_2O_2 treatment, condensed nuclei and active caspase-3, which are hallmarks of apoptosis, were found exclusively in phospho-ERK-negative cells (Fig. 9*B*). These data indicate that by 1 h, the cells have segregated into two populations and suggest that those that activate p53, a signaling molecule upstream of caspase-3, will proceed to apoptosis and those that activate ERK/*egr1* will maintain homeostasis.

Activation of p53 in Response to Oxidative Stress Downregulates ERK—Our results demonstrated that H_2O_2 activates both ERK and p53 in PC-12-D_2R cells. To elucidate the mechanism underlying these signaling pathways, we studied the cross-talk between ERK and p53 signaling mechanisms. The phosphorylation of ERK in response to H_2O_2 was blocked by using the selective inhibitor of ERK, PD98059 (56). In Western immunoblots using phospho-ERK and phospho-p53 antibodies, it was determined that addition of PD98059 (100 μM) 1 h before H_2O_2 treatment prevented the phosphorylation of ERK (Fig. 10*A*). However, PD98059 did not affect the phosphorylation of p53 in response to H_2O_2 (Fig. 10*A*).

To examine the effect of p53 on the activation of ERK, we

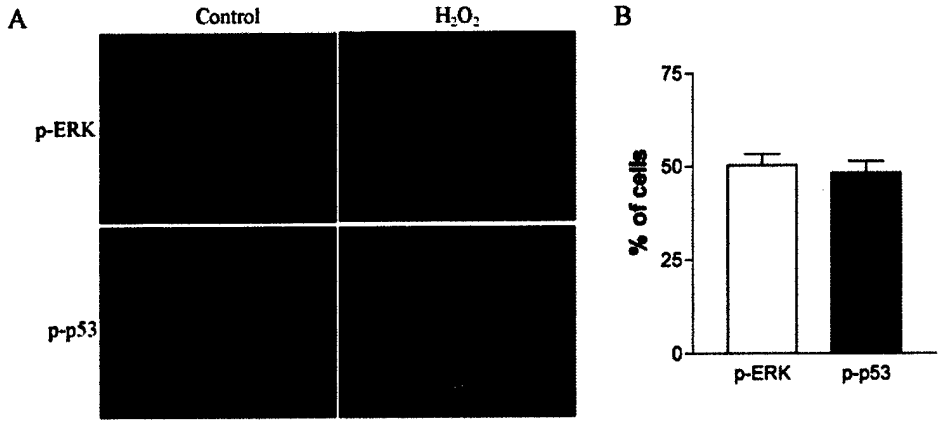

FIG. 4. **ERK and p53 activation in response to H_2O_2.** *A*, immunofluorescence microscopy of control PC-12-D_2R cells or cells treated with H_2O_2 (200 μM) for 1 h. Cells were labeled for phosphorylated ERK (*p-ERK*; *red*, *top*) or phosphorylated p53 (*p-p53*; *red*, *bottom*). The nuclei were counterstained with DAPI (*blue*). *B*, percentage of PC-12-D_2R cells showing phospho-ERK or phospho-p53 in response to H_2O_2 (200 μM) for 1 h. Cells showing fluorescence phospho-ERK (*p-ERK*) or phospho-p53 (*p-p53*) antibodies was scored as phospho-ERK or phospho-p53, respectively, in five experiments. Values represent the mean ± S.E. (200–400 cells scored per experiment).

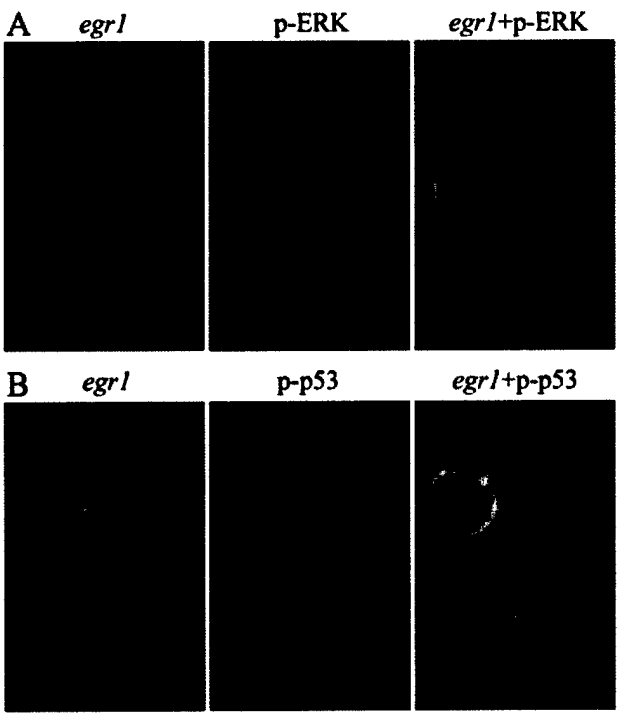

FIG. 5. **Oxidative stress activates ERK and p53 in different populations of cells.** *A*, FISH-immunofluorescence microscopy of PC-12-D_2R cells labeled with egr1 mRNA (*green*) and anti-phospho-ERK (*p-ERK*; *red*). *B*, PC-12-D_2R cells labeled with egr1 mRNA (*green*) and anti-phospho-p53 (*p-p53*; *red*). Cells were incubated with 200 μM H_2O_2 for 1 h. Note that egr1 induction and ERK phosphorylation occur within the same individual cells, whereas egr1 induction and p53 phosphorylation are mutually exclusive. The experiments were repeated four times with similar results.

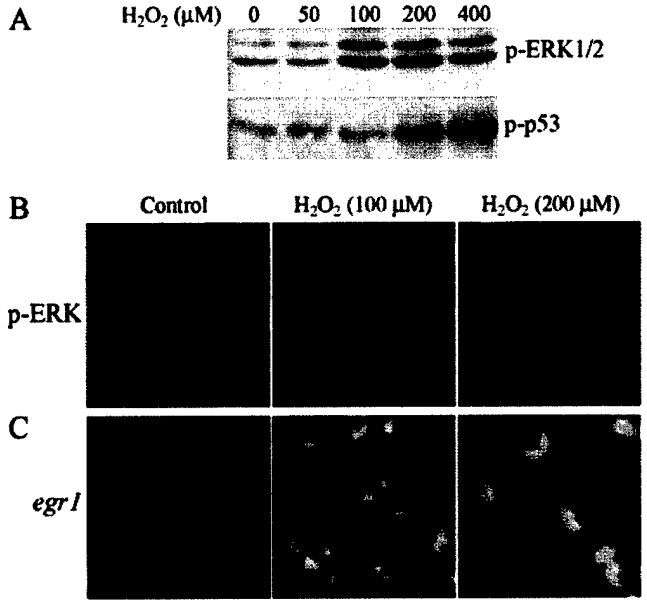

FIG. 6. **Concentration-dependent H_2O_2 activation of ERK and p53 in undifferentiated PC-12-D_2R cells.** *A*, cells were treated with the concentrations of H_2O_2 indicated for 1 h. Aliquots of cell extracts were then subjected to immunoblot analysis using antibodies against phospho-ERK (*p-ERK*) or phospho-p53 (*p-p53*). The experiments were repeated three times with similar results. *B*, concentration-dependent activation of ERK at single cell level. Immunofluorescence microscopy of active-ERK (*red*) in control cells and cells treated with 100 or 200 μM H_2O_2 for 1 h. *C*, concentration-dependent induction of *egr1* at single cell level. FISH for *egr1* (*green*) in control, cells treated with 100 or 200 μM H_2O_2 for 1 h. The nuclei were counterstained with DAPI (*blue*). The experiments were repeated three times with similar results.

used the p53 inhibitor pifithrin-α, which blocks p53 transcriptional activation and subsequent apoptosis (57). In Western immunoblots using phospho-ERK and phospho-p53 antibodies, we have found that pifithrin-α augmented the activation of ERK in presence of H_2O_2 (Fig. 10*B*). These results suggest that H_2O_2-induced ERK phosphorylation is negatively regulated by activation of p53. However, we found that 40 μM pifithrin-α did not inhibit the phosphorylation of p53 in response to H_2O_2.

To confirm the regulation of ERK by p53, we reduced the levels of p53 expression in PC-12-D_2R cells using RNA interference. After transfection with p53-specific or control siRNA, cultures were assessed for p53 mRNA expression by qPCR. As shown in Fig. 10*C*, p53 expression was substantially repressed (~4-fold) by 48 h after transfection. The involvement of p53 in oxidative stress-induced ERK activation was studied in PC-12-D_2R cells transfected with p53 or control siRNA. After 48 h of transfection, the cells were incubated with H_2O_2 and assessed for the phosphorylation of ERK. In control siRNA-transfected cells, H_2O_2 activated ERK similar to that observed in cells not transfected with siRNA (Fig. 10*D*). However, p53 repression by siRNA augmented the ERK phosphorylation (Fig. 10*D*). These results suggest that cells that activate p53 in response to oxidative stress suppress the activation of ERK.

DISCUSSION

In this study, we demonstrate that within the first hour of cells' exposure to oxidative injury, they activate specific signaling pathways that indicate whether the cells will ultimately succumb to or tolerate the insult. ERK activation by oxidative stress marks cells that have chosen to maintain homeostasis. In contrast, cells that activate p53 proceed to cell death. Our data indicate that ROS-mediated anti- and pro-apoptotic signaling events are triggered in each cell early after exposure to oxidative stress. These responses are sustained and mutually exclusive. These early, non-overlapping single cell responses are observed in both proliferating and differentiated PC-12-D_2R cells and in immortalized dopaminergic SN4741 neurons.

We find that activation of ERK and the induction of *egr1* within 1 h mark cells destined to survive after the initial oxidative insult. In PC-12 cells, ERK is mainly activated by growth factors and has been shown to be associated with cell proliferation, differentiation, and promotion of cell survival (13, 16, 58, 59). H_2O_2 increased the mRNA expression of the ERK-dependent genes *egr1*, c-*jun*, c-*fos*, and *mkp1*. These genes are known to be transcriptionally activated by increased cellular oxidation (16, 43, 44) and by nerve growth factor (46, 60). Evidence for an antiapoptotic role for ERK has been reported in PC-12 cells after growth factor withdrawal (13, 59) and exposure to oxidative stress (16). ERK has also been reported to function as a suppressor of ROS in superior cervical ganglion neurons (61). Activation of the ERK via the Ras/Raf/MEK pathway has further been shown to support survival of neurons in the nervous system (49, 50).

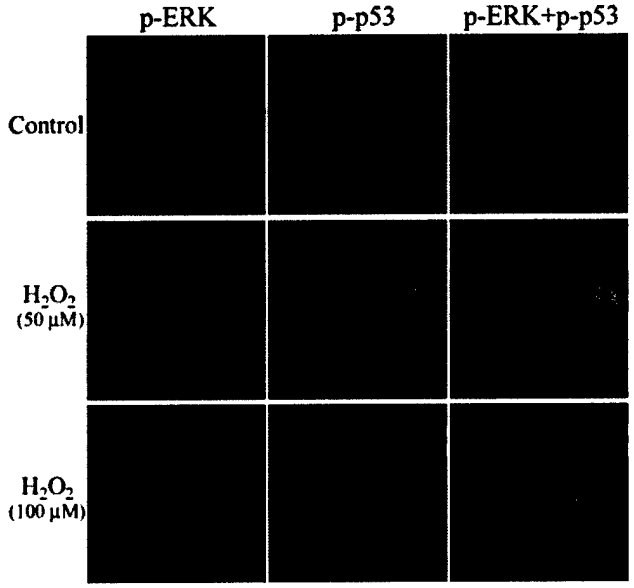

FIG. 7. **Concentration-dependent H_2O_2 activation of ERK and p53 in SN4741 neuronal cells.** Double-immunofluorescence microscopy of anti-phospho-ERK (*p-ERK*; *red*) and anti-phospho-p53 (*p-p53*; *green*) in untreated cells, cells treated with 50 μM H_2O_2, or cells treated with 100 μM H_2O_2 for 1 h. The nuclei were stained with DAPI (*blue*). Phospho-p53 is concentrated in the nucleus, whereas phospho-ERK is both cytoplasmic and nuclear. Note that phospho-p53 and phospho-ERK are not co-activated within the same individual cells. The experiments were repeated three times with similar results.

In contrast to the ERK response, we find that the early activation of p53 by H_2O_2 predicts the later induction of caspase-3 and apoptosis. The inability of a sublethal dose of H_2O_2 to activate p53 supports the involvement of p53 in apoptosis in these cells. The p53 tumor suppressor protein has been proposed as a key mediator of stress responses because it plays an essential role in the death of many cell types, including neurons (for review, see Ref. 62). Exposure to ROS can cause nuclear DNA double-stranded breaks that are detected by enzymes from the phosphoinositide 3-kinase family (63), resulting in phosphorylation of serine 15 of p53 and its consequent stabilization and accumulation (40, 41, 54). It has been suggested that modification of this serine regulates p53 stability by altering Mdm2-p53 interactions (40). Activation of p53 results in the up-regulation of proteins implicated in apoptosis (such as pro-apoptotic BAX and caspase-3) in many experimental systems (25, 51, 52, 64).

We demonstrate that activation of the ERK signaling pathway in response to ROS has no effect on the phosphorylation of p53, whereas p53 inhibition leads to ERK activation. It has been reported previously that ERK activation in response to cisplatin in ovarian cancer cells can phosphorylate p53 *in vitro* (65). In our experimental system, the pharmacological inhibition of ERK did not affect phosphorylation p53 in response to H_2O_2. In contrast, we found that inhibition of p53 by pifithrin-α or repression of p53 by siRNA augmented the activation of ERK by H_2O_2. These results suggest the existence of a negative signaling cross-talk pathway from p53 to ERK. This ERK suppression pathway most likely contributes to the structure of a signaling network switch that forces the cell to rapidly select among these two mutually exclusively patterns of response to oxidative stress.

We find that the recruitment of cells to the response state marked by phospho-p53 increases as the concentration of H_2O_2 increases and that the early response bifurcation is observed equally in dividing and NGF-differentiated cells. These findings suggest that the initial conditions of the cells that present opposite responses to oxidative stress are likely to be similar.

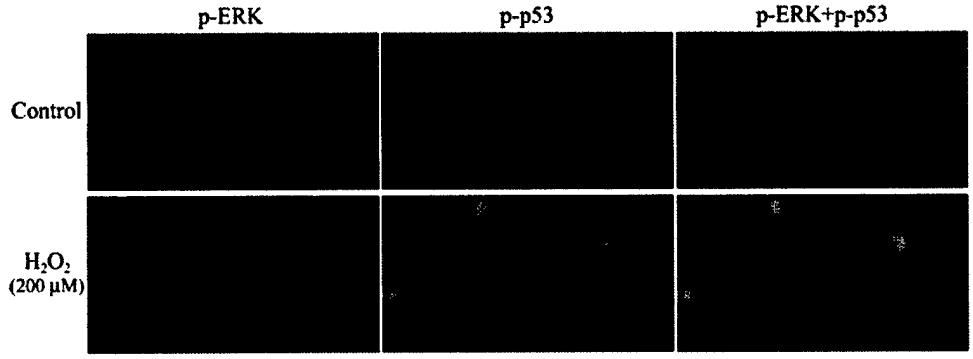

FIG. 8. **Oxidative stress activates ERK and p53 in different sub-populations of neuronally differentiated PC-12-D_2R cells.** Nerve growth factor-differentiated PC-12-D_2R cells were incubated with or without 200 μM H_2O_2 for 1 h and labeled with anti-phospho-ERK (*p-ERK*; *red*) and anti-phospho-p53 (*p-p53*; *green*) antibodies. The nuclei were counterstained with DAPI (*blue*). *Top frames*, control; *bottom frames*, H_2O_2 (200 μM; 1 h). Note that phospho-p53 and phospho-ERK are not co-activated within the same individual cells. The experiments were repeated four times with similar results.

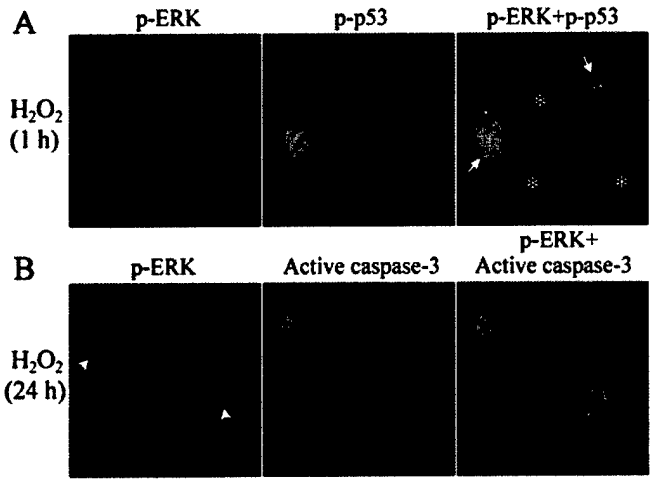

FIG. 9. **p53 activation correlates with caspase-3 mediated apoptosis.** *A*, double-labeling for phospho-ERK (*p-ERK*; *red*) and phospho-p53 (*p-p53*; *green*) in PC-12-D_2R cells incubated with 200 μM H_2O_2 1 h. *Asterisks* indicate cells with activated ERK and no phospho-p53. *Arrows* identify cells with phospho-p53 (*green*) with no phospho-ERK. *B*, double-labeling of phospho-ERK (*red*) and active-caspase 3 (*green*) in PC-12-D_2R cells incubated with 200 μM H_2O_2 for 24 h. *Arrows* indicate apoptotic cells with condensed nuclei. Active caspase-3 was localized exclusively in cells with condensed nuclei that are phospho-ERK-negative. The experiments were repeated three times with similar results.

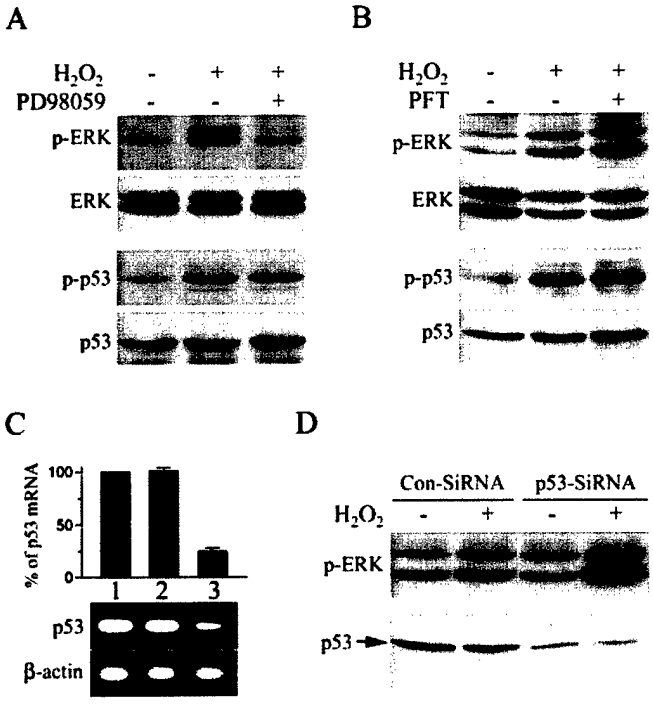

FIG. 10. **Cross-talk between p53 and ERK in response to oxidative stress.** *A*, PC-12-D_2R cells were pretreated with or without 100 μM PD98059 for 1 h and incubated with 200 μM H_2O_2 for 1 h. *B*, cells were pretreated with or without 40 μM pifithrin-α for 1 h and incubated with 200 μM H_2O_2 for 1 h. In *A* and *B*, the blots were stripped and reprobed using the antibodies recognizing total ERK or p53 proteins. *p-ERK*, phospho-ERK; *p-p53*; phospho-p53. *C*, repression of p53 mRNA by p53-siRNA. The cells were transfected with control or p53-siRNA, RNA was isolated after 48 h, and p53 mRNA levels were determined by qPCR (*graph*) or by reverse transcription PCR for 30 cycles and analyzed on agarose gel electrophoresis (*bottom*). β-Actin from the same samples was amplified as control. *Lane 1*, control; *lane 2*, control siRNA; *lane 3*, p53 siRNA. *D*, repression of p53 augmented the phosphorylation of ERK in response to H_2O_2. 48 h after transfection with control or p53-siRNA, cells were untreated or treated with H_2O_2 (200 μM) for 1 h and cell extracts were prepared. Equal amounts of protein were subjected to immunoblot analysis using phospho-ERK or p53 antibodies. The experiments were repeated three times with similar results.

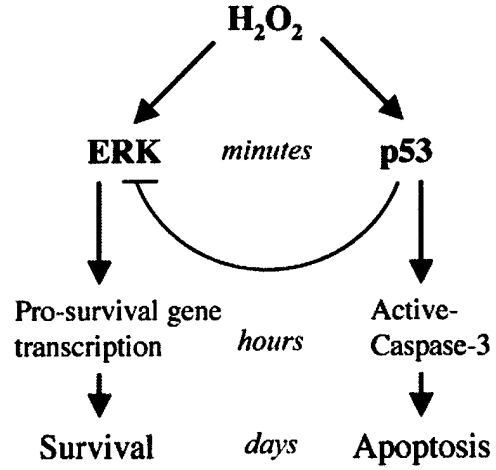

FIG. 11. **Model of H_2O_2-induced survival and apoptosis of PC-12-D_2R cells.** H_2O_2-induced ERK functions as antiapoptotic signal to maintain homeostasis of PC-12-D_2R cells, whereas p53 activity causes inhibition of ERK and functions as pro-apoptotic signal. The opposite effects of ERK and p53 are brought about by suppression of ERK activation by p53, resulting in an early divergence of the single cell responses toward survival or apoptosis.

For the individual cell, the choice between these two competing and mutually exclusive response states is stochastic. This model, we suggest (Fig. 11), is analogous to the random and exclusive divergence of initially pluripotential cells during development. We propose that the divergent outcome results not from initial differences in the state of the cells but from random selection forced by the design of the oxidative stress signaling circuits. This distinction is important in developing therapeutic strategies to intervene in this process. Our results and interpretation suggest that attempts to identify differences between the subset of cells that survive and those that succumb may be fruitless. On the other hand, further elucidating the structure of the signaling network switch responsible for this bi-stability and determining when the response state becomes unalterable are likely to help in devising rationale strategies to improve the odds for survival of an individual cell.

It has been hypothesized that ROS activates contradictory signaling pathways and that the dynamic balance between these pathways may be important in determining whether a cell survives or undergoes apoptosis (for review, see Ref. 12). Many studies using biochemical assays of cell homogenates have identified the concomitant activation of both pro- and antiapoptotic responses that have served as the basis for models of the underlying mechanisms (*e.g.* see Refs. 13, 66–69). We find that in PC-12-D_2R and SN4741 cells, even at early time points, that divergent responses do not coexist within the same cells. Activation of effector caspases is considered the final step in many apoptosis pathways. Consistent with our findings, it has been reported that caspase activation during apoptosis occurs in an all-or-none fashion (70). Studies using biochemical assays that monitor the average cellular response may obscure the actual decision-making signaling mechanisms by detecting simultaneous responses that are in fact segregated within different cell subpopulations. Our data suggest that "to die or not to die" is a question resolved quickly by each individual cell. Each individual cell rapidly responds to stress and achieves a coherent physiological state directed toward either apoptosis or survival.

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Central Role of p53 in Divergent Neurotoxin Responses

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Abstract

Distinct classes of neurotoxins such as the structural analogues of dopamine (6-hydroxydopamine), the mitochondrial complex I inhibitors (1-methyl-4-phenylpyridinium ion and rotenone) and the proteasome inhibitors (MG132 and lactacystin) all induce loss of dopaminergic neurons in experimental models that closely resemble Parkinson's disease in humans. Although neurotoxins mimicked dopaminergic neuronal loss in various experimental models, diverse molecular mechanisms have been implicated in the loss of neurons. We used mouse immortalized substantia nigral dopaminergic SN4741 neurons, rat pheochromocytoma cells and rat primary mesencephalic neuronal cultures as model cell systems for investigating neurotoxin induced early signaling mechanisms. In this pathway, the tumor suppressor protein, p53 is phosphorylated at serine15 site, a pattern predicted to cause cell death in various experimental systems. All neurotoxins tested caused activation of p53 and loss of cell viability, however only 6-hydroxydopamine and 1-methyl-4-phenylpyridinium ion caused casapse-3 activation. Consistent with caspase-3 activation, in 6-hydroxydopamine treated cells the phosphorylated p53 was localized in the nucleus and induced p53-dependent PUMA gene expression. The translocation of phopshorylated p53 into cytoplasm in response to proteasome inhibitor, MG132 correlated with its inability to activate caspase-3 and PUMA induction. However, the protection observed with inhibition of p53 by pifithrin alpha against neurotoxins further confirmed that p53 activation is critical for the cell loss in response to neurotoxins. Our results suggest that p53 acts as a common mediator for divergent neurotoxins to induce cell death by a caspase-dependent or -independent pathway.

Introduction

Parkinson's disease (PD) is characterized by preferential degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and a loss of striatal dopamine. However, the molecular mechanisms mediating degeneration of midbrain dopamine neurons in PD are poorly understood. Distinct classes of neurotoxins: structural analogues of dopamine (such as 6-hydroxydopamine (6-OHDA)), mitochondrial complex I inhibitors (such as 1-Methyl-4-phenylpyridinium ion (MPP^+) and rotenone), and proteasome inhibitors (such as lactacystin and MG132) all induce loss of dopaminergic neurons in experimental models that closely resemble Parkinson's disease in humans. However, several studies focusing on the mode of cell death induced by these neurotoxins have presented contrasting results (Lotharius et al., 1999; Nicotra and Parvez, 2000; Beal, 2001; Blum et al., 2001; Speciale, 2002; Han et al., 2003a).

6-OHDA is a selective catecholaminergic neurotoxin that is not only used as a pharmacological agent able to trigger PD-like stigmata (Blum et al., 2001; da Costa et al., 2003) but also likely corresponds to a natural dopaminergic catabolite that accumulates in Parkinson's disease-affected brains (Baba et al., 1998) and that appears to strongly contribute to this pathology (Goedert, 2001). Earlier studies have shown that 6-OHDA is transported into dopaminergic neurons where it is oxidized to produce hydrogen peroxide, superoxide, and hydroxyl radicals (Cohen and Heikkila, 1974; Graham et al., 1978). Mitochondrial dysfunction has also been linked to the pathogenesis of PD. Evidence for the involvement of mitochondria in PD comes from the finding that MPP^+ (1-methyl-4-phenyl-2, 3-dihydropyridinium), the active metabolite of the parkinsonism toxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), acts as a complex I inhibitor (Burns et al., 1983; Nicklas et al., 1985). 6-OHDA has also been shown to inhibit mitochondrial Complex I and IV *in vitro* (Glinka and Youdim, 1995). Recent reports suggest that the environmental toxin, rotenone that block mitochondrial complex I can also induce many characteristics of PD, including selective nigrostriatal dopaminergic degeneration, formation of ubiquitin and α-synuclein-positive nigral inclusions, and motor deficits (Betarbet et al., 2000; Alam and Schmidt, 2002; Sherer et al., 2002; Greenamyre et al., 2003; Sherer et al., 2003).

In sporadic PD, the levels of oxidatively damaged proteins and protein aggregation are elevated in the SNc and in other brain regions (Yoritaka et al., 1996; Alam et al., 1997; Lopiano et al., 2000). Recent genetic and molecular evidence suggests that defects in ubiquitin-proteasomal processing contribute to the accumulation of aberrant proteins may play a major role in the pathogenesis of PD. McNaught *et al.*, demonstrated structural and functional defects in 26/20S proteasomes in the SNc in sporadic PD postmortem brains (McNaught et al., 2002a; McNaught and Olanow, 2003). Further, it has been showed that the proteasome inhibition cause selective degeneration of dopaminergic neurons in fetal rat ventral primary mesencephalic cultures (McNaught et al., 2002c) as well as *in vivo* (McNaught et al., 2002b; McNaught et al., 2004).

The exact cause of this neuronal loss in PD is still unknown, but human postmortem studies have suggested that, nigral DA neurons die by apoptosis (Anglade et al., 1997; Kingsbury et al., 1998; Tatton et al., 1998; Hartmann and Hirsch, 2001). The neurotoxins belonging to distinct classes have been shown to produce reactive oxygen species and to inhibit mitochondrial complex I, as well as to mimic many behavioral, pharmacological, and pathological symptoms of this disorder (for review see Refs. (Beal, 2001; Blum et al., 2001; Speciale, 2002). Despite these parallels, the molecular mechanisms by which these neurotoxins kill cells remain unclear.

Neurotoxins commonly used to induce experimental parkinsonian syndromes have been shown to exert their pro-apoptotic actions via activation of caspase-3-like proteases in neuronal *in vitro* models (Dodel et al., 1999; Lotharius et al., 1999; Chun et al., 2001; Ahmadi et al., 2003; Han et al., 2003b; Sawada et al., 2004). We have demonstrated that when cells are exposed to oxidative stress they activate early signaling mechanisms that directs towards either apoptosis or survival (Nair et al., 2004). In the present study, therefore, we attempted to examine early signaling pathways activated by distinct classes of neurotoxins. We present data suggesting that diverse neurotoxins act through a common signaling protein, the p53, to induce cell death in *in vitro* culture models of PD and inhibition of p53 activity significantly increased cell survival following neurotoxin treatment.

Materials and Methods

Cell and neuronal cultures

PC12-D_2R (Nair and Sealfon, 2003; Nair et al., 2004) cells were maintained in DMEM (Invitrogen) supplemented with 500 μg/ml G418, 10% horse and 5% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. The dopaminergic neuronal cell line SN4741 was cultured as described previously (Son et al., 1999). The SN4741 neuronal cell line was cultured at 33°C with 5% CO_2 in medium containing Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), 1% glucose, penicillin-streptomycin, and 2 mM L-glutamine. When cells were grown on cover glass, they were coated with polyornithine (1 mg/ml of 0.15 M boric acid buffer, pH 8.4) overnight at 33°C and washed three times with phosphate buffered saline before use. For differentiation, 1 day before any treatment, the SN4741 cells were shifted to a differentiation condition (i.e., nonpermissive temperature 38°C and reduction of fetal calf serum to 0.5%).

Neuronal-enriched cultures containing dopaminergic neurons were prepared from the ventral mesencephalon of fetuses (14–15 days gestation) obtained from Sprague–Dawley rats (Taconic Farms, NY) as described elsewhere (McNaught et al., 2002c). Cells were suspended in D-MEM; supplemented with 15% fetal bovine serum, 1 mM sodium pyruvate and 4 mM L-glutamine) and plated at a density of 10^4 cells/cm^2 on 17-mm poly D-lysine-coated glass coverslips in 12-well culture plates for immunocytochemistry and 2 x 10^6 cells/100 mm plates for Western immunoblotting. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C for 1 day, after which the culture medium was changed to defined serum-free DMEM–F12 medium (supplemented with 25 μg/mL insulin, 100 μg/mL transferrin, 60 μM putrescine, 20 nM progesterone and 30 nM sodium selenite), and cells grown for a further 6 days before use.

Treatments with neurotoxins, H_2O_2 and pharmacological inhibitors

All chemicals were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA) except lactacystin (Calbiochem, San Diego, CA) and pifithrin-alpha (A.G. Scientific, La Jolla, CA). For cytotoxicity analysis, SN4741 cells were plated at a density 5 x10^3 cells/well on 96-microwell cell-culture plates (in100 μl of medium) and grown for 24 h. PC12-D_2R cells were plated at a density 1x10^5 cells/well well on 96-microwell cell-culture plates (in100 μl of medium). On the following day, each PD-related neurotoxins was added as indicated. When differentiated SN4741 cells were used, the cells were maintained in RF medium containing 0.5% serum at 38°C. For neuroprotection studies, pifithrin alpha (PFT-α) was applied 1 h prior to neurotoxin treatment.

Celltiter-blue and MTT reduction cytotoxicity assays

Following various treatments, 20 μl of the celltiter-blue™ reagent (Promega, Madison, WI) was added to each wells and the plates were incubated for 4 h. The dark blue non-fluorescent resazurin present in the celltiter-blue reagent is converted in living cells to pink and highly fluorescent resorufin. Quantification was then carried out in a spectrofluorometer (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) by measuring the fluorescence at excitation/emission wavelengths of 570/590 nm. Data are expressed as a percentage of the vehicle treated controls, and values represent the means S.E.M. from eight microwells from each

of three independent experiments (n=24). MTT reduction assay was carried as described (Nair and Sealfon, 2003).

Immunoblot analysis

After treatment the cells were washed with ice-cold PBS and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal C630, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, 5 μg/ml aprotinin and cocktail of protease inhibitors (Roche Diagnostics, GmbH) at 4°C for 20 min. After centrifugation at 14,000 x g for 20 min at 4°C, protein contents were measured with a Bio-Rad protein assay kit. Equal amounts of proteins (50-100 μg) were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and detected by immunoblotting using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) system as described (Nair and Sealfon, 2003; Nair et al., 2004). The blots were then stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol for 30 min at 50°C and re-probed with respective antibodies. Primary antibodies used were a rabbit polyclonal anti-phospho ERK, ERK, phospho-p53 (Ser15), and p53 (1:1000; Cell Signaling, Beverly, MA).

Immunocytochemistry

PC12-D_2R cells growing on collagen coated cover glass and SN4741 and primary mesencephalic neurons growing on poly-ornithine coated cover glass were treated as indicated. The cells were fixed, permeabilized and incubated with the primary antibody at 4°C overnight as described (Nair et al., 2003; Nair et al., 2004). Primary antibodies used were a rabbit polyclonal anti- phospho-p53 (1:400; Cell Signaling), a mouse monoclonal anti-cytochrome *c* and mdm2 (1:800; BD Bioscience), a sheep polyclonal anti-tyrosine hydroxylase (Chemicon, Temecula, CA), and a rabbit polyclonal antibody that recognizes activated caspase-3 (1:200; Promega). After washes with PBS the cells were incubated at room temperature for 2 hr with Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:400; Molecular Probes, Eugene, OR) as indicated. The cells were washed three times in PBS and the nuclei were stained with 1 μg/ml (in PBS) of the fluorescent DNA dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min and then washed with PBS. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium. Cells were examined under an Olympus (BX65) upright epifluorescence microscope.

Gene expression analysis

Cells were treated with neurotoxins or vehicle for indicated period of time and total RNA was isolated using StrataPrep total RNA miniprep kit according to the manufacturer's protocol. To determine the expression of target genes, reverse transcription polymerase chain reaction (PCR) or quantitative real time polymerase chain reaction (qPCR) was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using SYBR-Green assay, as previously described (Nair et al., 2004). To amplify PUMA, we used 5'-CCTCAGCCCTCCCTGTCACCAG-3' (forward) and 5' -CCGCCGCTCGTACTGCGCGTTG-3' (reverse), for Bax, we used 5'-GCTGATGGCAACTTCAACTG-3' (forward) and 5'-GATCAGCTCGGGCACTTTAG-3' (reverse), and for β-actin, we used 5'-TCCTGTGGCATCCATGAACT-3' (forward) 5'-CCAGGGCAGTAATCTCTTTCTTCTG-3' (reverse) primers in 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The authenticity of the PCR products was verified by melting curve analysis and agarose gel

electrophoresis. The comparative cycle threshold (C_T) method was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantitation for any given gene, expressed as -fold variation over control (untreated cells), was calculated after determination of the difference between C_T of the given gene A and that of the calibrator gene B (β-*actin*) in treated cells ($\Delta C_{T1} = C_{T1A} - C_{TB}$) and untreated cells ($\Delta C_{T0} = C_{T0A} - C_{TB}$) using the $2^{-\Delta\Delta CT(1-0)}$ formula (Nair et al., 2004). The C_T values are means of triplicate measurements and the xperiments were repeated 4 times.

Statistical analysis

The data were analyzed using GraphPad Prism data analysis program (GraphPad Software, San Diego, CA). For the comparison of statistical significance between two groups, Student's *t* tests for paired and unpaired data were used. For multiple comparisons, one-way ANOVA followed by *post hoc* comparisons of the group means according to the method of Tukey was used. *p* values , <0.001 were considered significant.

Results

Cytotoxic effects of neurotoxins on dopaminergic neuronal cells

PC12 cells have dopamine, dopamine transporters and monoaminooxidase B (MAO-B) and have been extensively used to study the mechanism of action of the Parkinsonism-causing neurotoxins (Viswanath et al., 2001; Ryu et al., 2002; Tai et al., 2003; Biasini et al., 2004). Immortalized dopaminergic neurons SN4741 used in this study were established from fetal rat mesencephalon, providing an opportunity to compare effects of Parkinson-causing neurotoxins on undifferentiated and differentiated DA neurons (Son et al., 1999; Chun et al., 2001). As shown in Fig. 1, the SN4741 cells express markers well established to be specific to dopaminergic neurons, such as tyrosine hydroxylase (TH). The rat mesencephalic primary cultures containing dopaminergic neurons (Fig. 1) were also extensively used as a model system to study the molecular mechanism of dopaminergic cell death.

6-OHDA, the mitochondrial complex 1 inhibitors, and proteasome inhibitors are known to cause the death of dopaminergic neurons *in vitro* and *in vivo* and are widely used to model Parkinson's disease (Burns et al., 1983; Nicklas et al., 1985; Betarbet et al., 2000; Blum et al., 2001; da Costa et al., 2003; McNaught and Olanow, 2003; McNaught et al., 2004; Nakaso et al., 2004). Using these *in vitro* cell cultures, we first characterized the cytotoxic effect of PD-mimicking agents such as 6-OHDA, MPP^+, rotenone and proteasome inhibitors, such lactacystin and MG132. Cytotoxicity was assessed by both the celltiter-blue and MTT reduction assays. Both methods showed significant correlation. From a dose–response study with various concentrations ranging from 0 to 400 μM of all the four neurotoxins to SN4741 cells, ~50–60% of cell survived at 25 μM 6-OHDA, 200 μM MPP^+, 1 μM rotenone and 5 μM MG132 after 24 h (Fig. 2B-E). Differentiated SN4741 cells (38°C) showed increased toxicity to MPP^+ and rotenone as compared to non-differentiated cells. Typically, at these concentrations a time dependent decrease in the number of surviving cells was observed (data not shown). We have also examined the cytotoxic effects of neurotoxins in another dopaminergic cell line, PC12-D_2R. Treatment with 6-OHDA, rotenone and MG132 induced significant cell death in a dose-dependent manner (Fig. 2G-I). Approximately 50–60% of cell survived at 100 μM 6-OHDA and 1 μM rotenone after 24 h and 5 μM MG132 after 48 h (Fig. 2I). Since neurotoxins are implicated in the generation of reactive oxygen species, we have also examined the toxic effects of H_2O_2 in these cells as a positive control (Fig. 2A and F).

Differential activation of early signaling mechanisms by various neurotoxins

The molecular events responsible for the loss of dopaminergic neuron in the substantia nigra pars compacta (SNc) in PD remain poorly understood. Moreover, the initial cause or causes and molecular mechanisms leading to the DA cell death are unknown. Recently we have demonstrated that when cells are exposed to oxidative stress they activate opposing early signaling mechanism such as extracellular-regulated kinase (ERK) and p53 and that may induce downstream signaling cascades to mediate whether the cell will succumb to stress or survive (Nair et al., 2004). We found that in PC12-D_2R cells, the toxic concentrations of 6-OHDA activated ERK and p53 (Fig. 3A), whereas, rotenone and MG132 only activated p53 within 1 h and 3 h, respectively (Fig. 3B, C). In primary mesencephalic neurons H_2O_2 (200 μM) or100 μM 6-OHDA activated ERK and p53 within 1 h of toxin exposure (Fig. 3D, E). Consistent with the p53 activation observed in PC12-D_2R cells, MG132 only activated p53 in primary neurons (Fig.

3F). Co-incubation of H_2O_2 and MG132 activated both ERK and p53 suggested that the activation of early signaling pathways is specific to individual neurotoxins (Fig. 3G). Interestingly, a marked decrease in cell viability was observed in cells treated with toxic concentrations of neurotoxins for 24 hr. However, virtually no cells loss was observed at the time points required to activate ERK or p53 by neurotoxins. These results suggest that toxins may regulate the state of phosphorylation of the ERK and p53 rapidly without significantly affecting the cell viability. Our data suggest that to mediate the toxic effects, distinct classes of neurotoxins differentially activate the early signaling mechanisms such as ERK and p53 in dopaminergic cells.

Differential modulation of p53 by distinct neurotoxins

The results provided in the report suggest that diverse neurotoxins activate p53 very early after toxin exposure. To underlie the mechanism by p53 in mediating the toxic effects of neurotoxins, we examined the localization of neurotoxin-activated p53 in dopaminergic cells. Activation of p53 can mediate apoptosis by transcriptional activation of pro-apoptotic genes like the BH3-only proteins Noxa and Puma (Villunger et al., 2003). However, p53 translocation to mitochondria also induces apoptosis by a transcription-independent signaling mechanism (Mihara et al., 2003). We show that, in primary cultures of dopaminergic neurons, PC12-D_2R and SN4741 cells treated with 6-OHDA, p53 displays a nuclear localization pattern after 1h of toxin exposure (Fig.4, 5A and B). Moreover, the p53-dpendent transcription of the BH3-only protein PUMA (p53 up-regulated modulator of apoptosis) is induced by H_2O_2 in PC12-D_2R cells (Fig. 6). However, in primary cultures of dopaminergic neurons, PC12-D_2R and SN4741 cells treated with MG132, p53 displays a cytoplasmic localization pattern (Fig. 4 5B) and failed to induce PUMA gene expression in these cells (Fig. 6B). These results suggest that diverse neurotoxins differentially modulate p53 and provide a molecular basis for the activation of different downstream signaling pathways causing the cell death observed in reported studies.

Neurotoxins activate different downstream signaling mechanisms to induce cell death

The differential modulation of p53 activity by distinct classes of toxins suggests that activation of different downstream signaling mechanisms may be responsible for the cell death in these cells. Previous studies suggest caspase-3 activation to be a mechanism mediating neurotoxicity in dopaminergic cells such as PC12 cells, SN4741, and primary cultures of mesencephalic neurons (Qiu et al., 2000; Chun et al., 2001; Bilsland et al., 2002; Ahmadi et al., 2003; Han et al., 2003b; Nair et al., 2003). In addition, activated caspases have been identified in the nigrostriatal region of animals and humans with Parkinson's disease (Hartmann et al., 2000; Mogi et al., 2000; Viswanath et al., 2001). Using caspase-3 immunocytochemistry, we observed that in PC12-D_2R cells caspase-3 was activated by 6-OHDA (Fig. 7A), whereas, in SN4741 cells both 6-OHDA and MPP^+ activated caspae-3 (Fig. 7B). The caspase-3 activation was only observed in cells with morphological features of apoptosis such as fragmented nuclei. The neurotoxicants rotenone and MG132 treatment caused nuclear fragmentation but failed to activate caspase-3 in these cells. These results suggest that various neurotoxins recruit distinct cell death mechanisms to cause cell loss.

To determine whether cytochrome *c* release accompanies p53-induced cell death, the cells were immunostained using anti-cytochrome *c* antibody. Cells treated with toxic concentrations of neurotoxins showed diffuse cytochrome *c* distribution throughout the cytosol,

but control cells maintained the typical mitochondrial distribution of cytochrome *c* (Fig. 7A and B), indicating that cytochrome *c* release from mitochondria is an event induced during neurotoxin-mediated cell death. This finding is in agreement with previous reports showing the mitochondrial dysfunction in various experimental models of PD.

p53 inhibition prevents neurotoxin induced cell loss

The tumor suppressor protein, p53 is a transcription factor that induces growth arrest or apoptosis in response to a variety of stress signals (Vogelstein et al., 2000; Sharpless and DePinho, 2002). Reactive oxygen species have been implicated in DNA damage by genotoxic agents. Phosphorylation at serine 15 of the p53 tumor suppressor protein is induced by DNA damage (Shieh et al., 1997; Lakin and Jackson, 1999). Several mechanisms for stress-induced p53 phosphorylation have been identified including *Ataxia Telangiectasia* mutated (ATM) kinase (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998; Waterman et al., 1998). To probe the mechanism of neurotoxin-mediated p53 phosphorylation, we treated cells with caffeine, an inhibitor of ATM kinase (Chen et al., 2003). We observed phosphorylation of p53 at serine15 in response to H_2O_2 that was significantly inhibited by caffeine in a dose-dependent manner (Figs. 8). Thus, neurotoxin-mediated ATM kinase activation appears to be one response of cells to oxidative stress.

To determine the role of rapid p53 activation in response to neurotoxins, we tested the p53 inhibitor pifithrin alpha under the above described neurotoxicity paradigms. Pre-treatment (1 h) of cells with pifithrin alpha demonstrated the significant neuroprotection against distinct neurotoxins by reducing the loss of cell viability in a dose-dependent manner in SN4741 and PC12-D_2R cells (Fig. 10). The significant neuroprotection was observed at concentrations of 2 μM and above and the maximal response observed with this inhibitor was at 10 μM concentration. The neuroprotective function of pifithrin alpha was further confirmed in comparison with increasing concentration of neurotoxins (Fig. 11). This indicates that inhibition of p53 alone is sufficient to prevent the toxicity of neurotoxins in these culture systems. Our results suggest that early p53 activation leads to cell death and inhibition of p53 can be neuroprotective.

Discussion

The diverse groups of neurotoxins such as 6-OHDA, MPP^+, rotenone, lactacystin, and MG132 all are known to induce Parkinsonism in experimental models of PD. However, several studies focusing on the mode of cell death induced by these neurotoxins have presented contrasting results (Lotharius et al., 1999; Nicotra and Parvez, 2000; Beal, 2001; Blum et al., 2001; Speciale, 2002; Han et al., 2003a). The PD mimetics, rotenone and *N*-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) or its active derivative, MPP^+, is thought to induce oxidative stress and impair energy metabolism by reducing mitochondrial complex 1 activity (Nicklas et al., 1985; Sherer et al., 2003)The structural analogue of DA, 6-OHDA has also been shown to produce reactive oxygen species and to inhibit mitochondrial complex I, as well as to mimic PD (Glinka and Youdim, 1995). More recently, several lines of evidence have converged to suggest that failure of the ubiquitin-proteasome system to clear unwanted proteins (e.g. oxidatively damaged, mutant and misfolded proteins) plays a major role in the etiopathogenesis of Parkinson's disease (McNaught and Olanow, 2003). Further, it has been showed that the proteasome inhibition cause selective degeneration of dopaminergic neurons in fetal rat ventral primary mesencephalic cultures (McNaught et al., 2002c) as well as *in vivo* (McNaught et al., 2002b; McNaught et al., 2004). Despite these parallels, the molecular mechanisms by which these neurotoxins kill cells remain unclear.

The loss of dopaminergic neurons induced by distinct classes of neurotoxins in experimental models PD suggest that mitochondrial dysfunction, oxidative stress, proteasome dysfunction, excitotoxicity, and inflammation are involved in the pathogenesis of PD (reviewed in (Dauer and Przedborski, 2003)). Here we show that diverse neurotoxins act through a common signaling protein, the tumor supressor protein, p53, to induce cell death in *in vitro* culture models of PD. The toxicity of neurotxins for dopaminergic cells appears to be mediated by a caspase 3-dependent and –independent signaling pathways that are activated by the differential activation of p53, and inhibition of p53 is sufficient to protect these cells from the toxicity. Our results suggest that neurotoxins can cause cell loss, which results from the induction of a signaling event that can activate diverse downstream mechanisms causing the ultimate execution of the cellular changes characteristic of this process.

Recently, we have demonstrated that oxidative stress activate both antiapoptotic ERK phosphorylation and pro-apoptotic serine-15 phosphorylation at p53 (Nair et al., 2004). Based on our results on the activation of ERK and p53, neurotoxins can be classified into two groups: such as neurotoxins activate ERK and p53 or only p53. The group of toxins activates ERK and p53 later induced cytochrome-c release and caspase-3 activation. The toxins only activated p53 later induced cytochrome-c release and failed to activate caspase-3. As with the recent findings by others that redistribution of cytochrome c into the cytosol is not sufficient for and does not always result in caspase activation in these cells (Deshmukh and Johnson, 1998; Von Ahsen et al., 2000; Zhou et al., 2000; Torok et al., 2002). Neurotoxins commonly used to induce experimental parkinsonian syndromes, e.g., MPP^+ and 6-OHDA, have been shown to exert their proapoptotic actions via activation of caspase-3-like proteases in neuronal *in vitro* models (Dodel et al., 1999; Elkon et al., 2001). However, Lotharius and coworkers (1999) found no evidence for apoptosis in MPP^+ treated rat mesencephalic neurons (Lotharius et al., 1999). Since the degradation of p53 is controlled by the ubiquitin-proteasome system, proteasome inhibition may induce the mitochondrial dysfunction and induce apoptosis (Rodriguez et al., 2000). Consistent

with this hypothesis, the slow degradation of p53 has been associated with a decreased proteasome activity in neurodegenerative diseases (Duan et al., 2002).

The p53 tumor suppressor protein has been proposed as a key mediator of stress responses because it plays an essential role in the death of many cell types, including neurons (for review, (Morrison et al., 2003)). Exposure to ROS can cause nuclear DNA double-stranded breaks that are detected by enzymes from the phosphoinositide 3-kinase family, resulting in phosphorylation of serine 15 of p53 and its consequent stabilization and accumulation (Shieh et al., 1997; Lakin and Jackson, 1999). The activation of elements known to be relevant to DNA damage, ATM kinase and p53, have been implicated in a variety of models of neurodegenerative disease (Canman et al., 1998; Shi et al., 2004). In our cell culture system induction of p53 serine 15 phosphorylation was blocked by caffeine suggested that ATM kinase is one of the upstream kinase involved in the activation of p53. The cell death pathway activated by p53 proceeds predominantly via the mitochondrial death at pathway. p53 can mediate apoptosis by transcriptional activation of pro- apoptotic genes like the BH3-only proteins Noxa and Puma, Bax, p53 AIP1, Apaf-1, and PERP and by transcriptional repression of Bcl2 and IAPs (Villunger et al., 2003). Activation of p53 results in the up-regulation of proteins implicated in apoptosis (such as proapoptotic BAX and caspase-3) in many experimental systems (Cregan et al., 1999; Gu et al., 2004). The p53 also known to translocate directly into mitochondria and induces apoptosis by transcription-independent mechanism(Mihara et al., 2003; Chipuk et al., 2004). Consistent with differential translocation of p53, we found in response to 6-OHDA, p53 was localized in the nucleus and induced PUMA gene transcription. However, p53 was localized in the cytoplasm and failed to induce PUMA gene induction in MG132 treated cells. These results correlated with the differences in their capacity to activate ERK and caspase-3.

The main aspect of the work reported here is that various neurotoxins activate p53 in dopaminergic cells and p53 inhibition by PFT-α prevents the neurotoxicants -mediated cell-loss. PFT-α is a stable, water soluble inhibitor of p53-dependent apoptosis was identified that was also shown to reduce activation of p53-regulated genes and it was shown to protect mice from lethal genotoxic stress associated with cancer treatment without promoting the formation of tumors (Komarov et al., 1999). Subsequently, PFT-α has been shown to protect against doxorubicin-induced apoptosis in mouse heart (Liu et al., 2004), camptothecin-, ischemia-,and dopamine-induced apoptosis in neurons (Culmsee et al., 2001; Culmsee et al., 2003), cisplatin-induced apoptosis in cochlear and vestibular hair cells (Zhang et al., 2003), and endotoxin-induced apoptosis in liver tissue (Schafer et al., 2003). Recently, p53 inhibitors have been shown to be neuroprotective in *in vivo* experimental model of PD (Duan et al., 2002).

Our findings suggest that neurotoxins can cause neuronal loss, which results from sequential signaling events, from the inducer of cell death to the ultimate execution of the cellular changes characteristic of this process. The early responses such as ERK and p53 to various neurotoxins indicate that common early signaling pathways shared after different cellular insults that may diverge later to induce cell death. Identifying agents that influence the cell death decision making process long before the effector enzymatic cascades have been activated may be a more generally applicable and effective approach to preventing DA neuronal loss. Understanding the early decision making process of cells exposed to neurotoxic stresses will be important in developing effective therapy.

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Figure Legends

Figure 1. **TH immunostaining of immortalized substantia nigra dopaminergic (SN DA) SN4741 cell line and primary mesencephalic neurons.** *A*, SN DA cell line SN4741 and *B*, primary mesencephalic neuronal cultures were immunostained with TH polyclonal antibody. The nuclei were stained with DAPI (blue).

Figure 2. **Neurotoxins induces a dose-dependent cell death in dopaminergic cells.** *A-E*, Differentiated (38°C) and undifferentiated (33°C) mouse immortalized SN4741 cultures and *F-I*, rat dopaminergic PC12-D_2R cells were treated with indicated concentrations of neurotoxins for 24 h except MG132 for 48 h in PC12-D_2R cells. *A* and *F*, H_2O_2; *B* and *G*, 6-OHDA; C, MPP^+; *D* and *H*, rotenone; *E* and *I*, MG132. The cell viability was assessed by celltiter-blue fluorescence assay described under "Experimental Procedures." Data represent mean ± S.E.M. of three independent experiments, n=8.

Figure 3. **Overall response of early signal transduction pathways to neurotoxins.** *A-C*, PC-12-D_2R cells were incubated with indicated concentrations of 6-OHDA, rotenone for 1 h or MG132 for 4 h, respectively. *D-F*, The primary mesencephalic neurons were prepared from rat embryos as described under "Experimental Procedures." Neuronal cultures were treated with indicated concentrations of H_2O_2 or 6-OHDA, for 1 h or MG132 for 4 h, respectively. *G*, PC-12-D_2R cells were co-incubated with 5 μM MG132 and 200 μM H_2O_2. MG132 was added to the medium and incubated for 3 h before incubating with H_2O_2 for 1 h. Cell lysates were prepared and immunoblots were carried out using specific phospho-ERK or p53 phosphorylated on serine 15 antibodies. The blots were stripped and reprobed using the antibodies recognizing total ERK or p53 proteins. The blots shown are representative of four independent experiments with similar results. The H_2O_2 and 6-OHDA phosphorylated ERK and p53, whereas, rotenone and MG132 only phosphorylated p53 in these cells.

Figure 4. **Differential translocation of p53 in response to neurotoxins in PC12-D_2R cells.** PC12-D_2R cells were treated with H_2O_2 (200 μM), 6-OHDA (200 μM), rotenone (2 μM) or lactacystin (10 μM) for 1 h and 6 h. *A*, Double immuno-labeling for phospho-p53 (*green*) and cytochrome *c* (red) was carried out using specific antibodies. Nuclei were stained with DAPI (blue). In cells treated with H_2O_2 and 6-OHDA phopsho-p53 was localized in the nucleus. Cytoplasmic localization of phospho-p53 was observed in cells treated with rotenone and lactacystin. *B*, Immunoblots showing the phoshorylation of p53 at serine 15 by different neurotoxins for indicated period of time. The cell lysates were analyzed by Western blotting antibodies specific to p53 phosphorylated on serine 15. The blots were stripped and reprobed using the antibodies recognizing total p53. The experiments were repeated four times with similar results.

Figure 5. **Differential translocation of p53 in response to neurotoxins in SN4741 and dopaminergic neurons in mesencephalic primary cultures.** A, Differentiated SN4741 cells were treated with H_2O_2 (50 μM) for 1 h or MG132 (5 μM) for 3 h. Immuno-labeling for phospho-p53 (*green*) was carried out using p53 serine 15 specific antibodies. Nuclei were stained with DAPI (blue). In cells treated with H_2O_2, phopsho-p53 was localized in the nucleus. Cytoplasmic localization of phospho-p53 was observed in cells treated with

MG132. *B*, Primary mesencephalic neurons were treated with H_2O_2 (200 μM) for 1 h or MG132 (5 μM) for 3 h. Immuno-labeling for TH (green) and phospho-p53 (*red*) was carried out using TH and p53 serine 15 specific antibodies, respectively. In dopaminergic neurons treated with H_2O_2 phopsho-p53 was localized in the nucleus. Cytoplasmic localization of phospho-p53 was observed in cells treated with MG132. The experiments were repeated four times with similar results.

Figure 6. **Differential transcriptional activation of p53-dependent PUMA gene expression in response to H_2O_2 and MG132.** *A*, PC12-D_2R cells were un-treated or treated with 200 μM H_2O_2 for indicated period of time and the changes in PUMA and Bax gene expression levels were determined by reverse transcription PCR for 30 cycles and analyzed on agarose gel electrophoresis. β-actin from the samples was amplified as control (n=4). *B*, PUMA induction in response to H_2O_2 and MG132. PC12-D_2R cells treated for 4 h with 5 μM MG132 or 200 μM H_2O_2 in the presence or absence of 10 μM PFT. The PFT-α was added to the medium 1 h prior to the addition of H_2O_2. Total RNA was isolated and changes in PUMA mRNA expression were measured using qPCR analysis. Results are presented as –fold increase over control values using the 2 C_T formula. Data represent are mean ± S.E of one experiment repeated three times with essentially same results.

Figure 7. **Caspase 3-dependent and –independent cell death induced by diverse neurotoxins.** *A*, PC12-D_2R cells were treated with H_2O_2 (200 μM), 6-OHDA (200 μM), rotenone (2 μM) or lactacystin (10 μM) for 24 h. *B*, SN4741 cells treated with 6-OHDA (50 μM), MPP^+ (200 μM), rotenone (2 μM) or lactacystin (5 μM) for 24 h. Double immuno-labeling for caapase 3 (*green*) and cytochrome *c* (red) was carried out using specific antibodies. Nuclei were stained with DAPI (blue). Active caspase-3 was localized exclusively in cells with fragmented nuclei in PC12-D_2R cells treated with H_2O_2 or 6-OHDA and in SN4741 cells treated with 6-OHDA and MPP^+. The experiments were repeated four times with similar results.

Figure 8. **H_2O_2-induced p53 phosphorylation involves the ATM kinase.** PC12-D_2R cells were pre-treated for 1 h with the indicated concentrations of caffeine followed by incubation with 200 μM H_2O_2 for 1 h. After incubation, cell lysates were subjected to immunoblotting with antibodies specific for p53 or p53 phosphorylated on Ser-15. Blots are representative of three independent experiments.

Figure 9. **Effect of p53 inhibition on neurotxins-induced cell death.** *A*. p53 inhibitor, pifithrin alpha significantly increased cell survival against neurotoxins in a dose-dependent manner. Differentiated SN4741 cells were pre-treated with indicated concentration of pifithrin alpha for 1 h prior to the addition of 50 μM H_2O_2, 25 μM 6-OHDA, 200 μM MPP^+, 1 μM rotenone, or 5 μM MG132. The cell viability was assessed after 24 h. Data are expressed as a percentage of the vehicle treated controls, and the values represent the mean S.E.M from eight microwells from each of four independent experiments . * $P<0.01$ compared with respective toxins (H_2O_2, 53.02 ± 2.46; 6-OHDA, 70.91 2.10; MPP^+, 66.56± 1.47; rotenone, 60.21 ± 0.83; MG132, 50.00 ± 3.21) by ANOVA and Tukey's post-hoc comparison. *B*, Neuroprtection by p53 inhibition in PC12-D_2R cells against neurotoxicity. Cells were pre-treated with indicated

concentration of pifithrin alpha for 1 h prior to the addition of 200 μM H_2O_2, 200 μM 6-OHDA, 1 μM rotenone, or 5 μM MG132. The cell viability was assessed after 24 h except MG132 was assessed after 48 h. Data are expressed as a percentage of the vehicle treated controls, and the values represent the mean S.E.M from eight microwells from each of four independent experiments . * $P<0.01$ compared with respective toxins (H_2O_2, 62.62 ± 1.53; 6-OHDA, 62.39 ± 3.11; rotenone, 68.83 ± 3.71; MG132, 65.14 ± 0.85) by ANOVA and Tukey's post-hoc comparison. In SN4741 and PC12-D_2R, p53 inhibitor significantly increased cell viability in a dose dependent manner.

Figure 10. **The efficacy of neuroprotection by p53 inhibition is dependent on the concentration of toxin.** The differentiated SN4741 cells were pre-treated with or without 10 μM PFT-α for 1h prior to incubation with indicated concentrations of H_2O_2. The cell viability was assessed after 24 h. Data are expressed as a percentage of the vehicle treated controls, and the values represent the mean S.E.M from eight microwells from each of three independent experiments . * $P<0.01$ compared with respective concentrations of H_2O_2 in the absence of PFT-α (10 μM, 70.08 ± 1.81 vs 92.03 ± 3.79; 25 μM, 55.86 ± 1.14 vs 78.86 ± 5.85; 50 μM, 38.63 ± 1.65 vs 58.12 ± 0.96; 100 μM, 18.87 ± 0.75 vs 39.31 ± 1.44).

Figure 1

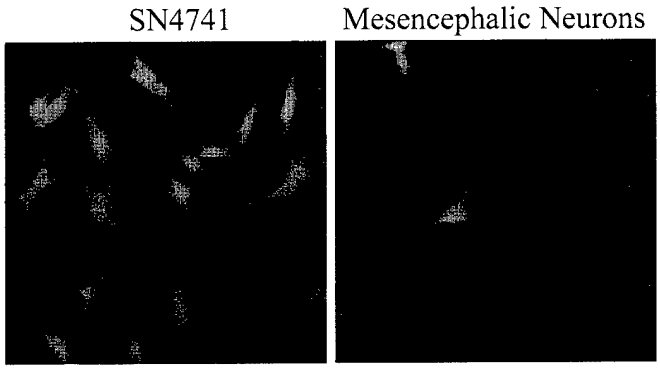


Figure 2

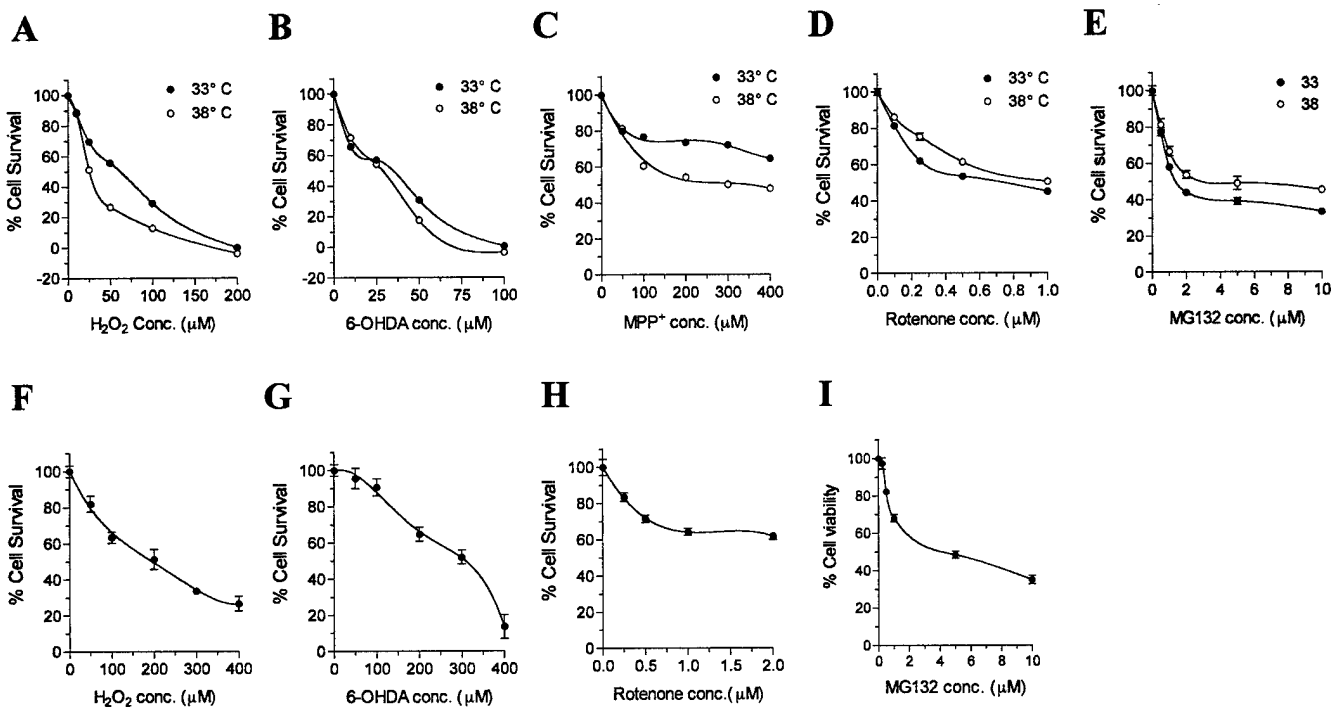

A
B
C
D
E
F
G
H
I
33° C
38° C
% Cell Survival
H_2O_2 Conc. (μM)
6-OHDA conc. (μM)
MPP^+ conc. (μM)
Rotenone conc. (μM)
MG132 conc. (μM)
% Cell survival
% Cell viability
H_2O_2 conc. (μM)
Rotenone conc.(μM)

Figure 3

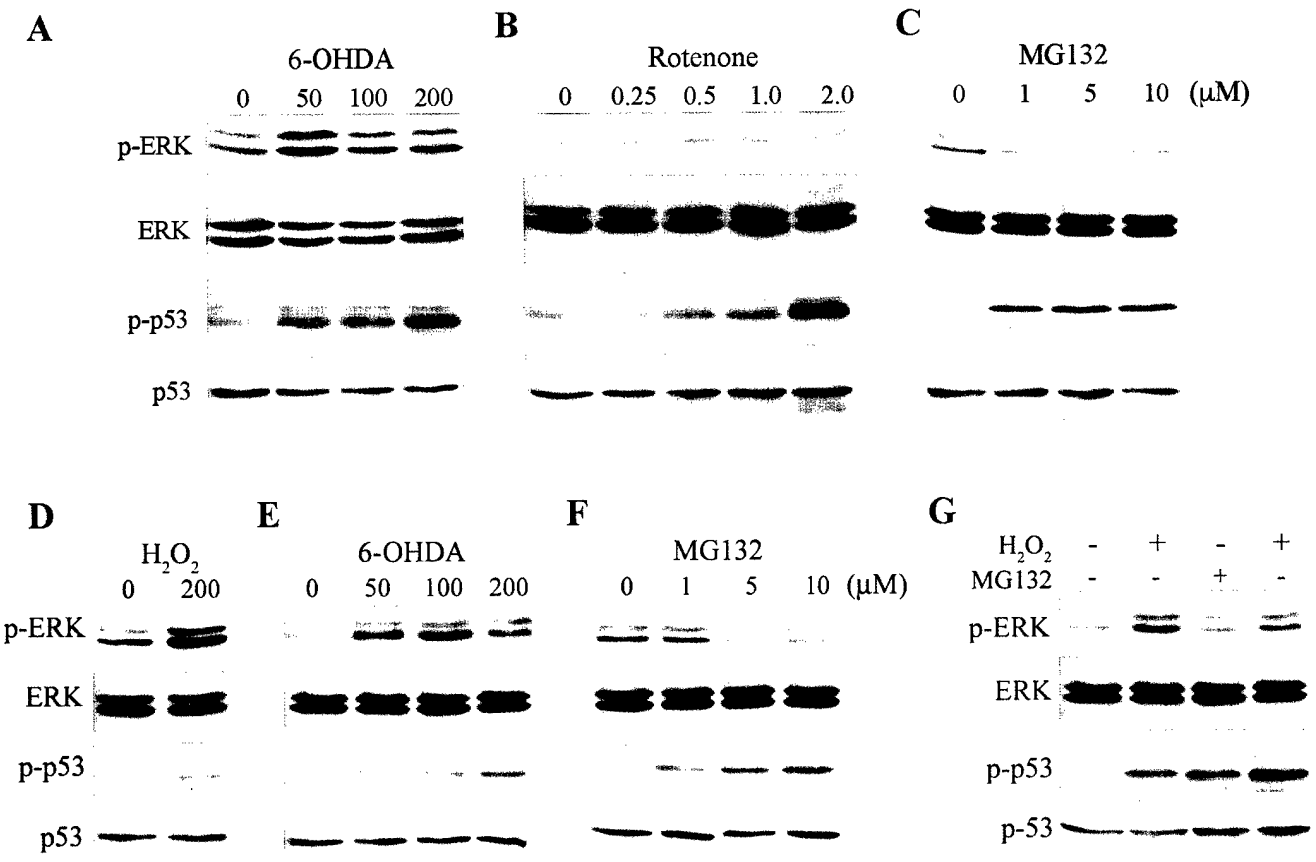

A
6-OHDA
0 50 100 200
p-ERK
ERK
p-p53
p53
B
Rotenone
0 0.25 0.5 1.0 2.0
C
MG132
0 1 5 10 (μM)
D
H2O2
0 200
p-ERK
ERK
p-p53
p53
E
6-OHDA
0 50 100 200
F
MG132
0 1 5 10 (μM)
G
H2O2 - + - +
MG132 - - + -
p-ERK
ERK
p-p53
p-53

Figure 4

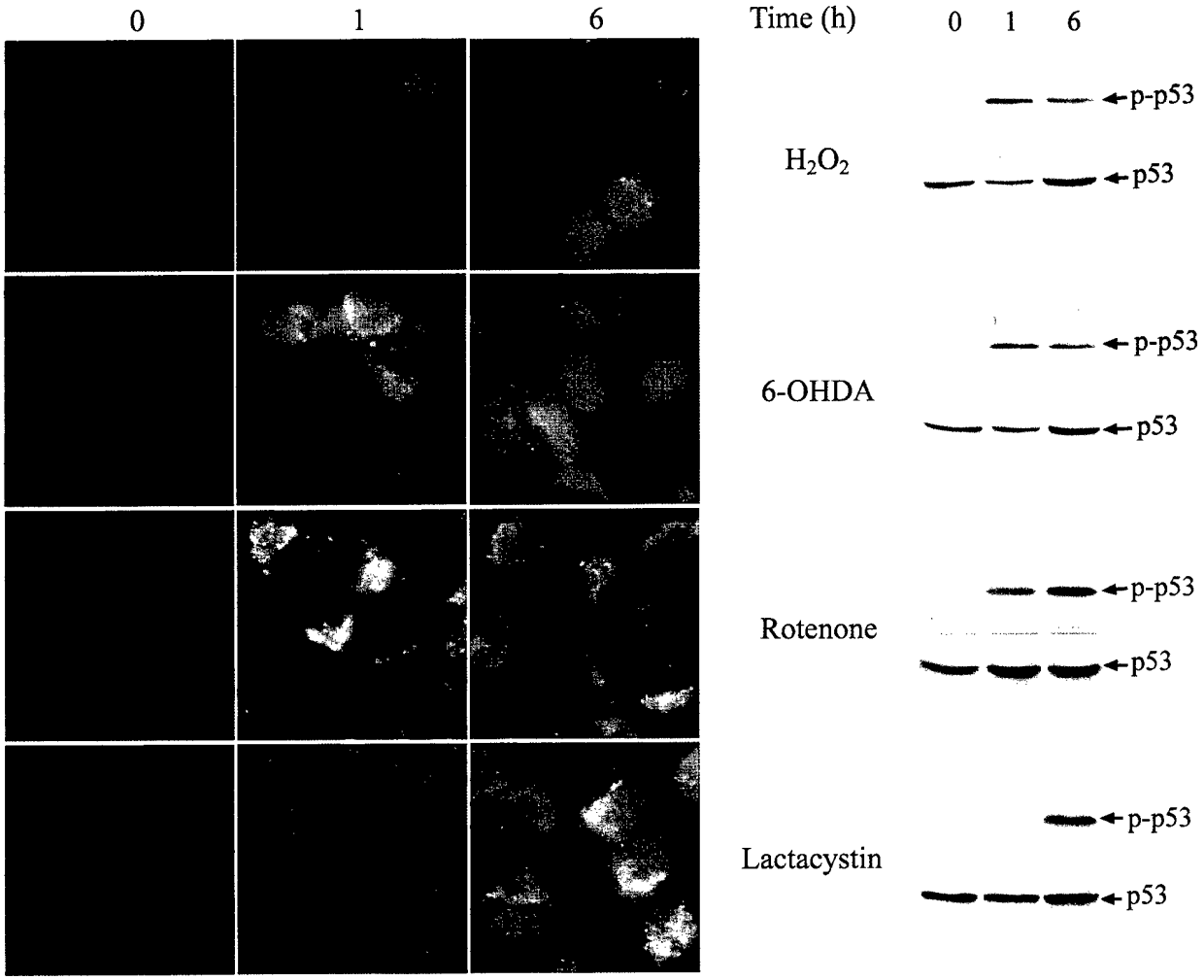

0
1
6
Time (h)
0 1 6
p-p53
H_2O_2
p53
p-p53
6-OHDA
p53
p-p53
Rotenone
p53
p-p53
Lactacystin
p53

Figure 5

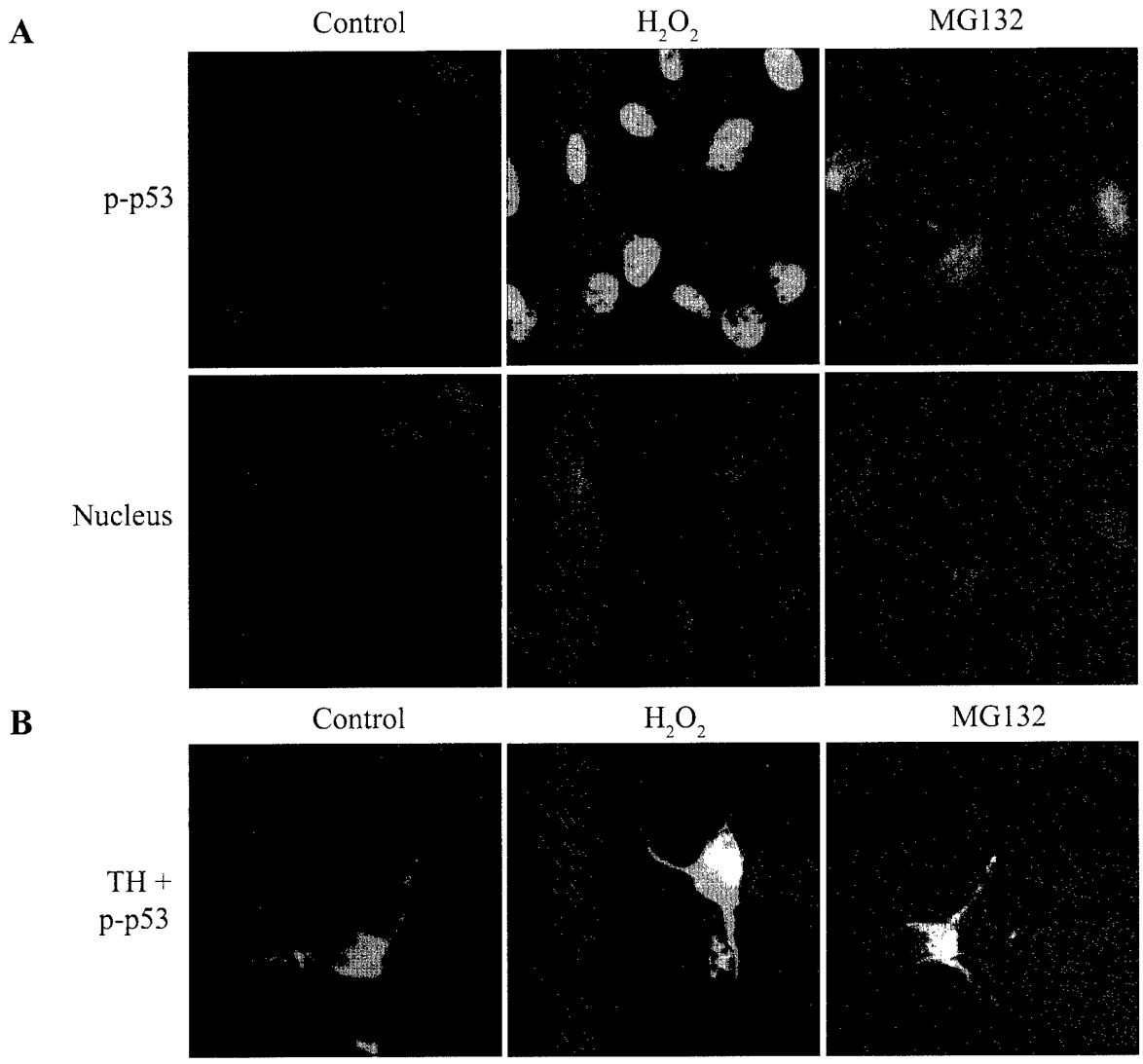

A
Control
H_2O_2
MG132
p-p53
Nucleus
B
Control
H_2O_2
MG132
TH +
p-p53

Figure 6

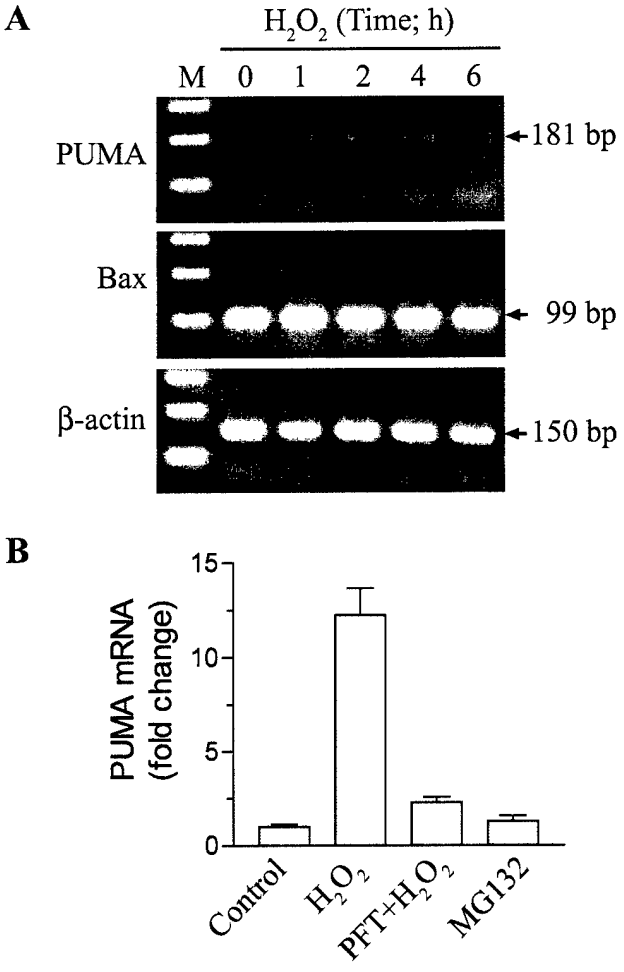

A
H_2O_2 (Time; h)
M 0 1 2 4 6
PUMA
181 bp
Bax
99 bp
β-actin
150 bp
B
PUMA mRNA (fold change)
15
10
5
0
Control
H_2O_2
PFT+H_2O_2
MG132

Figure 7

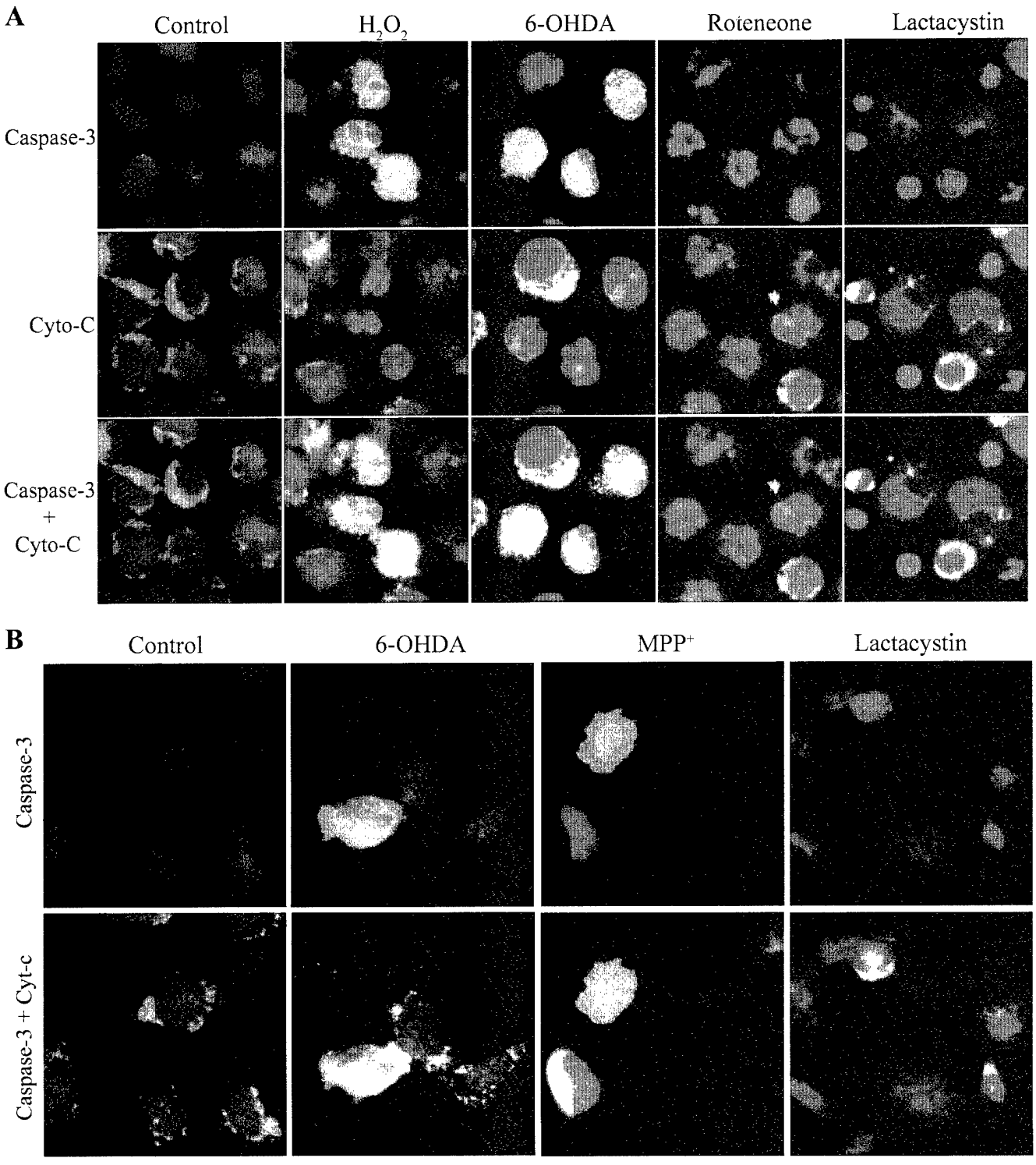

A
Control
H_2O_2
6-OHDA
Roteneone
Lactacystin
Caspase-3
Cyto-C
Caspase-3
+
Cyto-C
B
Control
6-OHDA
MPP+
Lactacystin
Caspase-3
Caspase-3 + Cyt-c

Figure 8

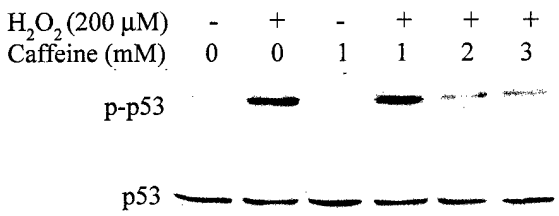
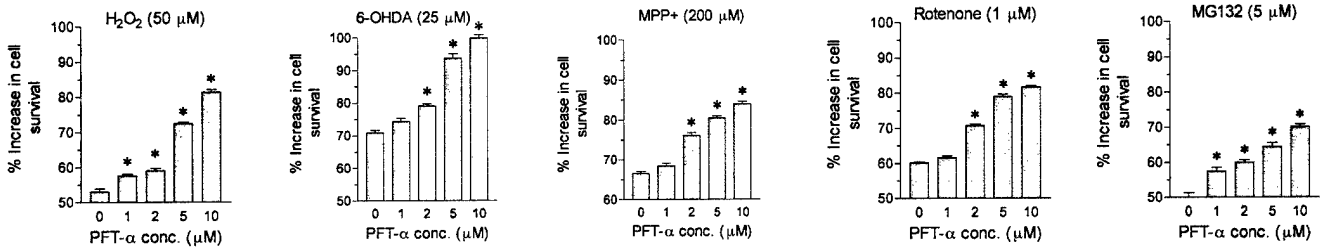


Figure 9

A. SN4741



B. PC12-D_2R

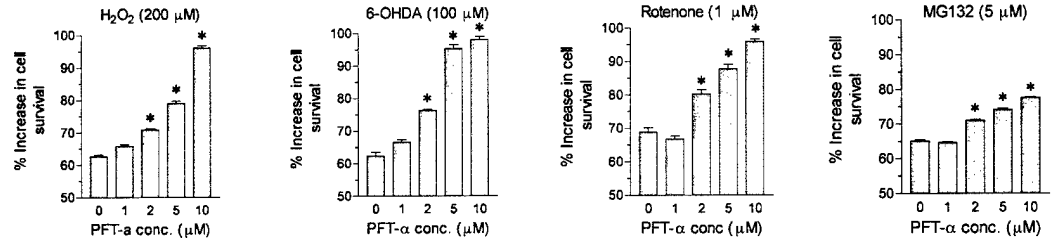
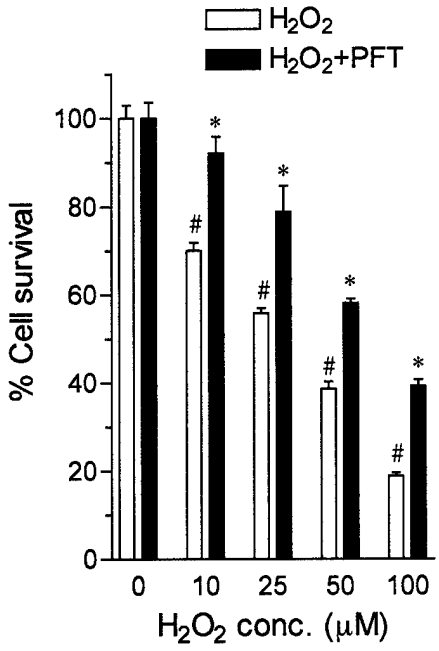


Figure 10


H2O2
H2O2+PFT
% Cell survival
100
80
60
40
20
0
0
10
25
50
100
H2O2 conc. (μM)
#
*

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Activation of phosphoinositide 3-kinase by D_2 receptor prevents apoptosis in dopaminergic cell lines

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Whereas dopamine agonists are known to provide symptomatic benefits for Parkinson's disease, recent clinical trials suggest that they might also be neuroprotective. Laboratory studies demonstrate that dopamine agonists can provide neuroprotective effects in a number of model systems, but the role of receptor-mediated signalling in these effects is controversial. We find that dopamine agonists have robust, concentration-dependent anti-apoptotic activity in PC12 cells that stably express human D_{2L} receptors from cell death due to H_2O_2 or trophic withdrawal and that the protective effects are abolished in the presence of D_2-receptor antagonists. D_2 agonists are also neuroprotective in the nigral dopamine cell line SN4741, which express endogenous D_2 receptors, whereas no anti-apoptotic activity is observed in native PC12 cells, which do not express detectable D_2 receptors. Notably, the agonists studied differ in their relative efficacy to mediate anti-apoptotic effects and in their capacity to stimulate [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) binding, an indicator of G-protein activation. Studies with inhibitors of phosphoinositide 3-kinase (PI 3-kinase), extracellular-signal-regulated kinase or p38 mitogen-activated protein kinase indicate that the PI 3-kinase pathway is required for D_2 receptor-mediated cell survival. These studies indicate that certain dopamine agonists can complex with D_2 receptors to preferentially transactivate neuroprotective signalling pathways and to mediate increased cell survival.

Key words: dopamine agonist, G-protein, neuroprotection, Parkinson's disease, signal transduction.

INTRODUCTION

Parkinson's disease (PD) is characterized by preferential degeneration of dopamine (DA) neurons in the substantia nigra pars compacta. Inhibition of oxidative phosphorylation, excitotoxicity and generation of reactive oxygen species are considered important mediators of neuronal death in PD [1]. Recent studies suggest that apoptosis may play a role in the loss of DA neurons in PD [2]. The major executioners of apoptosis, caspases, are activated in dopaminergic substantia nigra neurons from PD patients [3,4]. One experimental model for PD uses the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ('MPTP') to lesion DA neurons. Chronic administration of MPTP has been found to activate caspases [5,6] and to induce apoptosis in the substantia nigra pars compacta of mice [7].

Laboratory studies demonstrate that DA agonists can protect DA neurons in a variety of tissue culture and *in vivo* models of PD [8]. In the clinic, DA agonists have long been employed as an adjunct to levodopa therapy in advanced PD patients who experience motor complication [9]. Prospective double-blind clinical trials have also demonstrated that DA agonists can provide symptomatic benefits for early PD patients with a reduced risk of motor complications compared with levodopa [10,11]. Recent clinical trials have reported that, in comparison with levodopa, DA agonists delay the rate of decline in neuroimaging surrogate markers of nigrostriatal function [12]. These clinical trials raise the possibility that DA agonists may slow the rate of disease progression and are neuroprotective in PD. There is, however, uncertainty as to the mechanisms responsible for these effects and how they might be protective in PD. Proposed mechanisms include levodopa sparing, direct anti-oxidant effects, stimulation of auto-receptors and inhibition of subthalamic nucleus-mediated excitotoxicity [8]. In addition, some *in vitro* and *in vivo* studies have noted that the protective effects of DA agonists were eliminated when they were co-administered with D_2-receptor antagonists, suggesting that D_2-receptor activation may contribute to the neuroprotective effects observed in these models [13,14].

In order to clarify the contribution of the D_2 receptor to DA-agonist-mediated neuroprotection and to investigate the underlying mechanisms, we studied the effects of DA agonists in a PC12 cell line model system in the presence and absence of DA D_2 receptors. In these experiments, PC12 cells were induced to undergo apoptosis by either oxidative stress or trophic-factor withdrawal. We found that certain DA agonists, but not all, could induce a robust increase in cell survival via activation of the D_2 receptors. Furthermore our results implicate phosphoinositide 3-kinase (PI 3-kinase) in receptor-mediated cell survival and suggest a dissociation between neuroprotective signalling pathways and the G-protein activation classically associated with D_2-receptor signalling.

MATERIALS AND METHODS

Chemicals

[^{3}H]Spiperone (specific radioactivity 99.0 Ci/mmol) and [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]; specific radioactivity 1250 Ci/mmol) was from NEN (Boston, MA, U.S.A.). Bromocriptine, pergolide, quinpirole, *R*(+)-7-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetraline (7-OH-DPAT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Abbreviations used: PD, Parkinson's disease; DA, dopamine; PI 3-kinase, phosphoinositide 3-kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; NGF, nerve growth factor; ERK, extracellular-signal-regulated kinase; [^{35}S]GTP[S], [^{35}S]guanosine 5′-[γ-thio]triphosphate; 7-OH-DPAT, *R*(+)-7-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetraline; DMEM, Dulbecco's modified Eagle's medium; MPP^+, 1-methyl-4-phenylpyridinium.

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and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). LipofectAMINE™ and Dulbecco's modified Eagle's medium (DMEM) were obtained from Life Technologies (Gaithersburgh, MD, U.S.A.). Pramipexole was a gift from Pharmacia (Kalamazo, MI, U.S.A.). Protein kinase inhibitors PD98059, SB203580, LY294002 and wortmannin were from Calbiochem (La Jolla, CA, U.S.A.). Antibodies specific to phospho-extracellular-signal-regulated kinase (ERK)1/2, ERK1/2, phospho-p38 kinase and p38 kinase were from Cell Signalling Technology (Beverly, MA, U.S.A.). The anti-active caspase-3 antibody was from Promega (Madison, WI, U.S.A.) and anti-rabbit Cy3-conjugated antibody was from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Cell culture

PC12 cells [15] were maintained in DMEM supplemented with 10 % horse serum/5 % fetal bovine serum in a humidified atmosphere containing 5 % CO_2 at 37 °C. For differentiation, PC12 cells were plated on to collagen-coated plates in DMEM containing 10 % horse serum and 5 % fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in DMEM supplemented with 1 % fetal bovine serum and 100 ng/ml nerve growth factor (NGF) for 10–14 days. Nigral DA cell line SN4741 (a generous gift from Dr J. H. Son, Columbia University, New York, NY, U.S.A.) was cultured as described in [16].

Cell-viability assay

The MTT reduction assay is one of the most widely used assays for determining cell viability [17]; it detects living cells, but not dead ones, and the signal generated is dependent on the degree of activation of the cells [18]. PC12 or PC12-D_2R (PC12 cells stably transfected with the human DA D_2 receptor) cells were plated on 96-microwell cell-culture plates (4×10^3 cells/well in 100 μl of medium) and grown for 24 h. Thereafter, H_2O_2 was added either with or without other compounds at the indicated concentrations, and cells were incubated for another 24 h. When the immortalized dopaminergic cell line SN4741 was used the number of cells per well in a microwell plate was 1×10^4 and incubated with 50 μM H_2O_2 at 37 °C for 18 h. MTT solution (10 μl; 5 mg/ml in PBS) was added to the wells, containing 100 μl of medium, and the plates were incubated for 4 h. Thereafter, 100 μl of a solubilization solution (0.1 M HCl in absolute isopropanol) was added and incubated overnight to dissolve the water-insoluble formazan salt. Quantification was then carried out with an ELISA reader at 570 nm using a 655 nm filter as a reference. Data are expressed as a percentage of the untreated controls, and values represent the means ± S.E.M. from eight microwells from each of four independent experiments ($n = 32$).

Apoptosis analysis

Apoptosis was measured by nuclear DNA staining, caspase-3 immunocytochemistry and caspase-3 activity. Cells were exposed to 200 μM H_2O_2 and fixed by incubating in 4 % formaldehyde for 30 min. The cells were then permeabilized in PBS/0.2 % Triton X-100 for 10 min. The cells were incubated with blocking buffer (PBS/0.1 % Tween 20/5 % BSA) for 2 h at room temperature. Anti-active caspase-3 antibody (diluted 1 : 250) was added and incubated overnight at 4 °C. After washing, cells were incubated with donkey anti-rabbit Cy3-conjugate antibody (diluted 1 : 500) for 2 h at room temperature. The cells were washed twice in PBS and the nuclei were stained with 1 μg/ml of the fluorescent DNA dye DAPI (in PBS) for 10 min and then washed with PBS. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) mounting medium. For caspase-3 activity, 0.5×10^6 cells/100-mm-tissue-culture plates were grown for 24 h and treated with H_2O_2 as indicated. Active caspase-3 was measured using the Caspase-3/CPP32 fluorimetric assay kit (Biovision, Palo Alto, CA, U.S.A.). Enzymic activity was determined spectrofluorimetrically (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA, U.S.A.) by measuring the kinetics of fluorescence increase at excitation/emission wavelengths of 400/505 nm.

Plasmid transfection

To develop the PC12-D_2R cell line, the D_{2L} cDNA [19] was subcloned into pRC/RSV, transfected using lipofectAMINE into the PC12 cells and G418-resistant clones were isolated and screened for [^{3}H]spiperone ligand binding.

[^{3}H]Spiperone and [^{35}S]GTP[S] binding

[^{3}H]Spiperone and [^{35}S]GTP[S] binding assays were carried out essentially as described in [20]. Membranes were incubated with [^{35}S]GTP[S] (0.5 nM), GDP (5 μM) and increasing concentrations of DA agonists at 37 °C for 20 min. Data were analysed by non-linear regression analysis using the Inplot curve fitting program (GraphPad v3.0).

Immunoblotting

PC12-D_2R cells (3×10^6 cells/100-mm plate) were grown for 24 h and following respective treatments, the cells were washed twice with ice-cold PBS and lysed in buffer 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Igepal C630, 1 mM PMSF, 1 mM sodium orthovandate, 5 μg/ml aprotinin and a cocktail of protease inhibitors (Roche Diagnostics GmbH) at 4 °C for 20 min. After centrifugation at 14 000 g for 20 min at 4 °C, equal amounts of proteins were resolved by SDS/PAGE. The resolved proteins were electrotransferred to nitrocellulose membrane and incubated with phosphorylated ERK1/2 or p38 kinase antibodies, and then detected with peroxidase-conjugated secondary antibodies and chemiluminescent ECL reagent. The blots were then stripped in stripping buffer containing 62.5 mM Tris/HCl, pH 6.7, 2 % SDS and 100 mM β-mercaptoethanol and probed for total ERK or p38 kinase protein.

Statistical analysis

Data were analysed by either two-tailed Student's t test or ANOVA followed by Tukey's test for multiple comparisons.

RESULTS

Oxidative-stress-induced apoptosis in PC12 cells

We used oxidative stress or withdrawal of trophic support to induce apoptosis in PC12 cells as a model system for studying the putative neuroprotective property of D_2-receptor agonists. Cells were incubated for 24 h with varying concentrations of H_2O_2, and cell viability was assayed using the MTT metabolism assay.

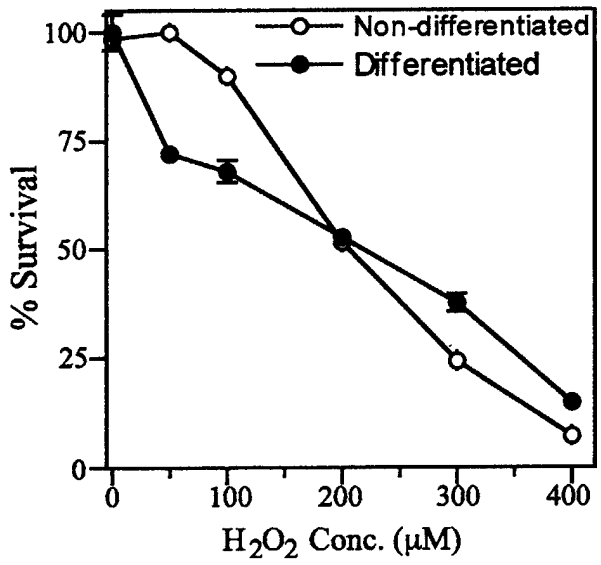

Figure 1 Concentration-dependent cell death induced by H_2O_2 in undifferentiated and differentiated PC12 cells

Cells were exposed to various concentrations of H_2O_2 for 24 h and cell viability was assessed using the MTT assay ($n = 8$).

Both undifferentiated and post-mitotic PC12 cells that had been differentiated into a neuronal phenotype by NGF exposure were studied. H_2O_2 reduced cell survival in a concentration-dependent manner (Figure 1). NGF-differentiated cells were more sensitive than undifferentiated cells to low H_2O_2 concentrations and were similar in sensitivity at higher H_2O_2 concentrations. In comparison with untreated controls, cell survival observed in differentiated cells exposed to 50 μM H_2O_2 was 72.1 ± 4.8 % compared with 99.9 ± 2.4 % in non-differentiated cells ($P < 0.001$). Cell survival observed with 200 μM H_2O_2 was 52.8 ± 2.2 and 51.5 ± 2.1 % in differentiated and undifferentiated cells respectively.

Apoptotic degeneration is associated with activation of caspase-3-like proteases in *in vitro* models of PD [6,21,22]. To test whether the oxidative-stress-initiated cell death was associated with apoptosis, we evaluated the effects of H_2O_2 on caspase-3 activation (Figure 2). Results obtained with enzymic caspase-3 assay show that 100 μM H_2O_2 activates caspase-3 as early as 2 h and that activity continued to increase until 16 h (Figure 2C). With 200 μM H_2O_2 treatment, maximal caspase-3 activation was observed at 6 h (Figure 2C). These patterns of active caspase-3 were consistent with the greater degree of cell loss observed with increasing concentrations of H_2O_2 (Figure 1). To confirm that caspase-3 activation in these cells was associated with morphological features of apoptosis, differentiated cells were labelled for caspase-3 activation and stained with the DNA dye DAPI. H_2O_2 at 200 μM induced the characteristic pattern of chromatin condensation in cells that also stained positive for caspase-3 activation (Figure 2A). Apoptotic changes were evident 8 h after the addition of H_2O_2, and were noted in 42.2 ± 6.9 % of nuclei after 16 h (Figure 2B).

Differentiated PC12 cells undergo apoptosis in the absence of NGF and serum [23]. After 24 h of trophic withdrawal, cell viability of differentiated PC12 cells was reduced by 48.5 ± 3.0% compared with control cells ($P < 0.01$). The trophic-withdrawn cells also stained positive for active caspase-3 and showed nuclear fragmentation (results not shown). Collectively, these data indicate that H_2O_2 or trophic withdrawal causes cell death in PC12 cells by inducing apoptosis.

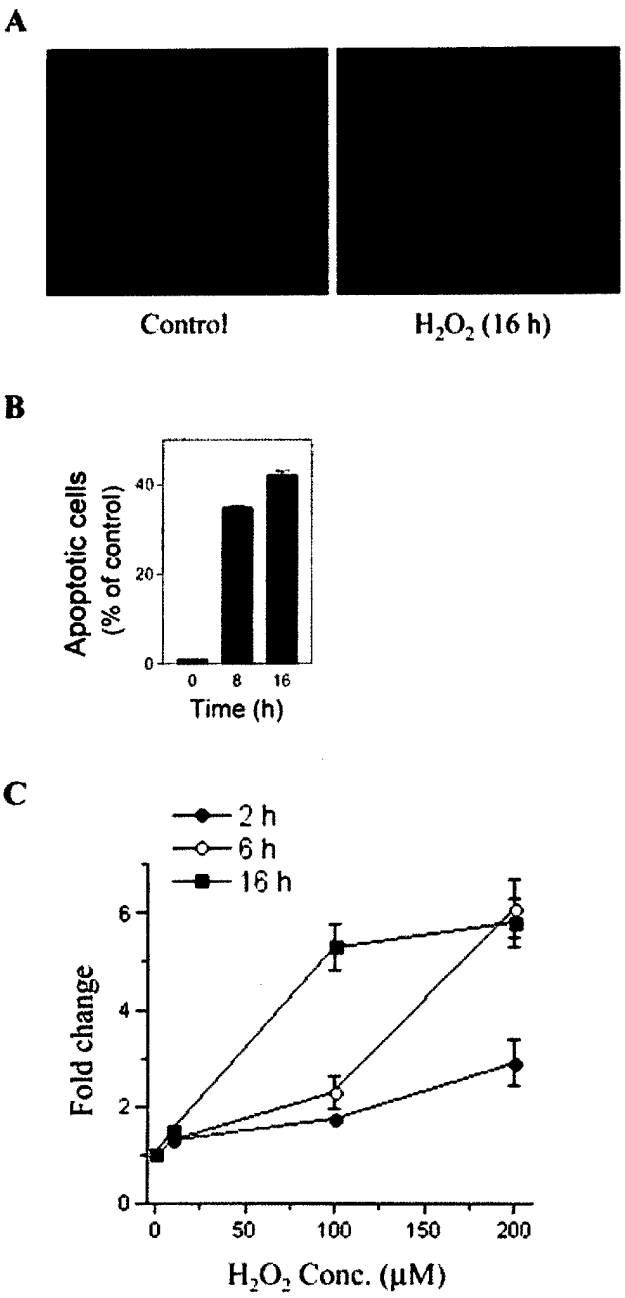

Figure 2 Oxidative-stress-induced apoptosis in PC12 cells (A) and caspase-3 activation induced by H_2O_2 in differentiated PC12 cells was associated with nuclear condensation

(**A**) The nuclei were stained blue (DAPI) and active caspase-3, red. Left-hand panel, control; right-hand panel, PC12 cells 16 h following 200 μM H_2O_2 exposure. Note the association of caspase-3 activation with chromatin condensation. (**B**) Percentage of apoptotic cells with condensed nuclei and active caspase-3 determined by counting random fields ($n = 6$) at 8 and 16 h following H_2O_2 exposure, which showed a significant difference ($P < 0.01$) compared with the control. (**C**) H_2O_2-induced caspase-3 enzymic activity in undifferentiated PC12 cells was dependent on H_2O_2 concentration and incubation period ($n = 4$).

D_2-receptor activation is required for the prevention of apoptosis in PC12 cells

We studied the potential of DA agonists to protect against apoptosis induced by oxidative stress or by serum and NGF withdrawal in PC12 cells that lacked D_2 receptors. The absence of D_2 receptors in these cells was demonstrated by the lack of specific

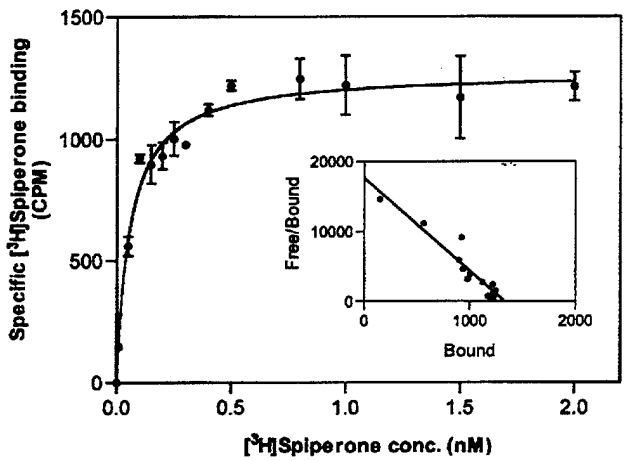

Figure 3 Saturation analysis for [^{3}H]spiperone binding in membranes of PC12-D_2R cells

D_{2L} DA receptors were expressed in PC12 cells and saturation binding analysis using [^{3}H]spiperone was performed to determine levels of D_2 receptors. Binding parameters (K_d and B_{max}) were derived from the data, and values are expressed as the mean ± S.D. values from one experiment performed in triplicate, representative of three independent experiments.

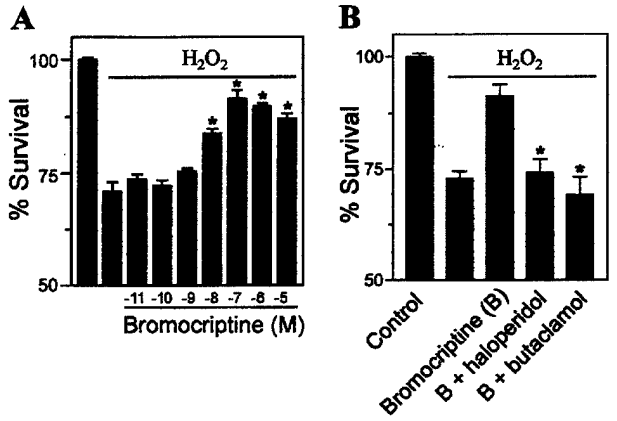

Figure 4 D_2-receptor activation protects PC12 cells from oxidative-stress-induced apoptosis

(**A**) Bromocriptine increased cell survival of undifferentiated PC12-D_2R cells exposed to H_2O_2. Cells were exposed to 200 μM H_2O_2 for 24 h in the presence of increasing concentrations of bromocriptine and cell viability assessed by MTT assay ($n = 8$). *$P < 0.01$ compared with H_2O_2 alone. (**B**) DA agonist neuroprotection depends on interaction with the D_2 receptor. The protective effect of bromocriptine against H_2O_2-induced apoptosis was eliminated in the presence of the D_2-receptor antagonists haloperidol or butaclamol (1 μM; $n = 8$). *$P < 0.01$ compared with bromocriptine + H_2O_2.

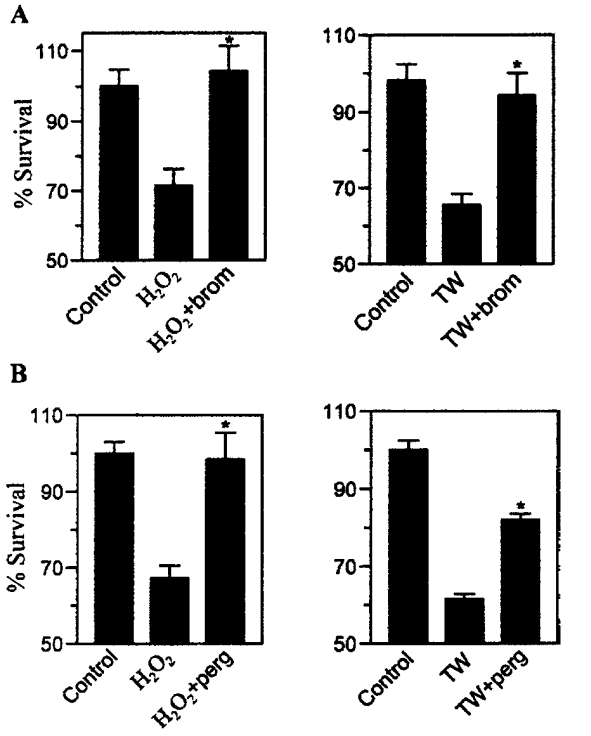

Figure 5 DA agonists protect against apoptosis in differentiated PC12-D_2R cells

Differentiated PC12-D_2R cells were exposed to 200 μM H_2O_2 or trophic withdrawal (TW) in the presence or absence of 100 nM bromocriptine (brom) and pergolide (perg; $n = 8$). *$P < 0.05$ compared with H_2O_2 or trophic withdrawal.

binding using the D_2-receptor ligand [^{3}H]spiperone as well as by the inability to detect D_2-receptor mRNA by PCR (results not shown). The DA agonists tested, bromocriptine and pergolide, provided no protection against apoptosis in either differentiated or undifferentiated cells exposed to H_2O_2 or removed from NGF and serum. These results indicate that these DA agonists were not neuroprotective in the absence of functional D_2 receptors in this model system.

To study the possibility that the DA D_2 receptor might mediate neuroprotection, PC12 cells were stably transfected with the human DA D_2 receptor (PC12-D_2R). PC12-D_2R membranes showed specific [^{3}H]spiperone binding with a B_{max} of 545 ± 14 fmol/mg of protein, a K_d of 0.54 ± 0.07 nM (Figure 3) and competitive binding K_i values for various D_2-receptor agonists and antagonists consistent with reported values for the human D_2 receptor [19]. In PC12-D_2R cells, oxidative stress or withdrawal of trophic support induced cell loss and the stigmata of apoptosis similar to what was observed in PC12 cells that lacked D_2 receptors.

Undifferentiated PC12-D_2R cells were exposed to 200 μM H_2O_2 for 24 h in the presence or absence of various concentrations of the DA agonist bromocriptine, and cell viability was measured. In contrast to the lack of agonist-mediated neuroprotection observed in the parent cell line, bromocriptine dramatically protected undifferentiated PC12-D_2R cells from H_2O_2-induced apoptosis in a robust and concentration-dependent manner (Figure 4A). The protective activity of DA agonists was inhibited by the D_2-receptor antagonists haloperidol and butaclamol, further confirming that the neuroprotection was mediated by activation of the D_2 receptor (Figure 4B). In NGF-differentiated cells, 100 nM bromocriptine and pergolide similarly inhibited apoptosis induced by H_2O_2 and trophic-factor withdrawal (Figure 5).

To explore whether the D_2-receptor activation protects dopaminergic neurons, we investigated the neuroprotection by bromocriptine in the mouse immortalized nigral DA cell line SN4741, which expresses tyrosine hydroxylase, the DA transporter and the D_2 auto-receptors [16]. It has been reported that treatment with 1-methyl-4-phenylpyridinium (MPP^+), neurotoxins and H_2O_2 equally induces oxidative-stress-dependent apoptotic cell death in SN4741 cells [16,24]. The DA agonist bromocriptine showed significant concentration-dependent increased cell survival against H_2O_2 (50 μM)-induced cell death in SN4741 cells (Figure 6). These results suggest that activation of an endogenous D_2 receptor can be neuroprotective.

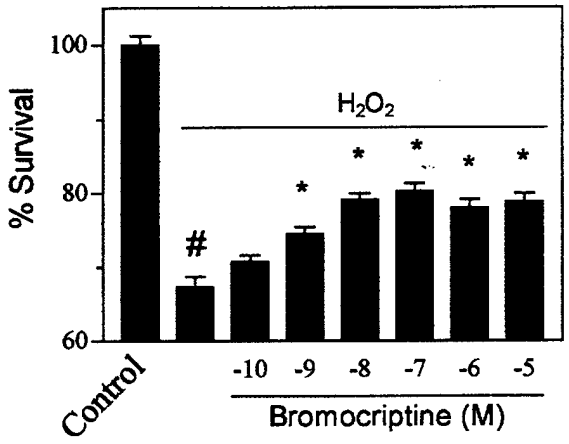

Figure 6 Bromocriptine protects nigral DA cell line SN4741 from oxidative-stress-induced cell death

Cells were exposed to 50 μM H_2O_2 for 18 h in the presence of increasing concentrations of bromocriptine and cell viability was assessed by MTT. Data are mean $\pm$ S.E.M. ($n = 16$). #$P < 0.001$ compared with control. *$P < 0.001$ compared with H_2O_2 alone.

Differential neuroprotective activity of DA agonists does not correlate with GTP[S] binding

DA agonists varied considerably in their ability to stimulate neuroprotection (Figure 7A). Bromocriptine provided the greatest neuroprotection in this assay, 7-OH-DPAT had low activity and pramipexole was nearly devoid of neuroprotective activity (Figure 7A). The major signalling pathway associated with activation of the D_2 receptor involves the heterotrimeric G-proteins, principally G_i. The capacity of agonists to stimulate G-protein activation was determined by [^{35}S]GTP[S] binding in membranes from PC12-D_2R cells (Figure 7B). Whereas bromocriptine and pramipexole showed marked differences in neuroprotective activity, they demonstrated similar agonist-stimulated [^{35}S]GTP[S] binding and they both exceeded the [^{35}S]GTP[S]-binding activity of pergolide and 7-OH-DPAT. These results indicate that DA agonists differ in their capacity to induce neuroprotection and further suggest that there is a weak correlation between activation of neuroprotective and G-protein signalling pathways.

Neuroprotection by D_2-receptor agonists involves the PI 3-kinase signalling cascade

To characterize the molecular mechanisms responsible for the D_2-receptor-mediated neuroprotection, we tested the effectiveness of several protein kinase inhibitors against bromocriptine-mediated cell survival in oxidative-stress-induced apoptosis. Inhibition of ERK by PD98059 (50 μM) or p38 kinase by SB203580 (20 μM) had no effect on the increased cell survival caused by D_2R activation (Figure 8 and results not shown). In contrast, the D_2 receptor-stimulated increase in cell survival required PI 3-kinase activation. The PI 3-kinase inhibitors wortmannin (100 nM) and LY294002 (10 μM) completely abolished the capacity of bromocriptine to protect against oxidative-stress-induced cell death (Figure 8). Control toxicity studies indicated that, at the concentrations used, the inhibitors had no effect on cell survival, as determined by MTT assays (results not shown). However, the inhibitors of ERK and p38 kinase completely inhibited the activation of ERK by epidermal growth factor and p38 kinase by NaCl, respectively (Figure 9). These data suggest that activation of the PI 3-kinase pathway through the

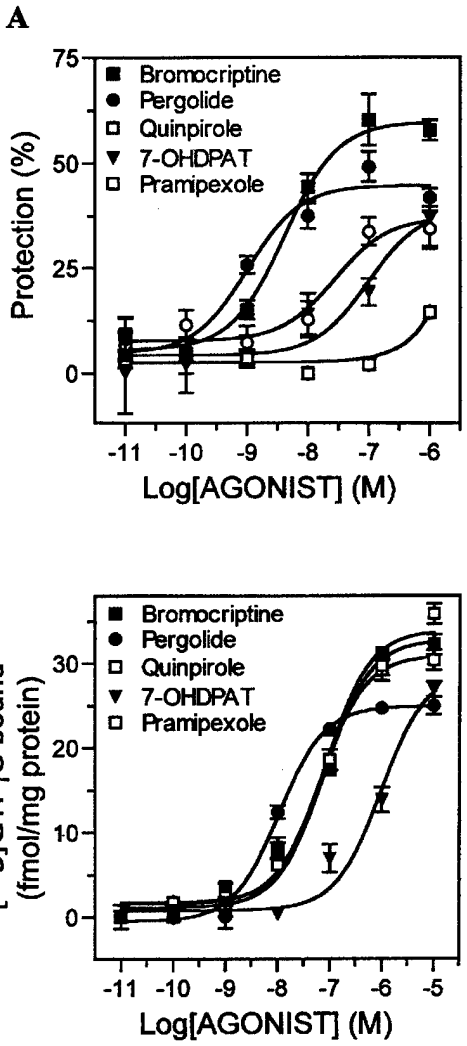

Figure 7 Differential neuroprotective efficacy of DA agonists

(**A**) Comparison of DA agonists in protection of PC12-D_2R cells against oxidative stress. Cells were exposed to 200 μM H_2O_2 for 24 h in the presence or absence of various concentrations of five DA agonists and the effects on cell survival determined by MTT assay. The rank order of protection observed was consistent in three independent experiments. (**B**) Concentration–response curve of the stimulation of [^{35}S]GTP[S]-specific binding to PC12-D_2R membrane by DA agonists. Data are means $\pm$ S.E.M. from one experiment performed in triplicate, representative of three independent experiments.

D_2 receptor contributes to the protection against oxidative-stress-induced apoptosis in PC12-D_2R cells.

DISCUSSION

Our results demonstrate that DA agonists can protect PC12 cells that express the human D_2 receptor and a nigral DA cell line, SN4741, from apoptosis induced by oxidative stress. Similar protective effects could not be obtained in PC12 cells that lacked D_2 receptors. These observations suggest that the D_2 receptor plays a critical role in the neuroprotective effects conferred by DA agonists in this model system. Furthermore, we find that specific DA agonists vary in their capacity to provide anti-apoptotic effects and that neuroprotective effects do not correlate closely with the capacity of the agonist to activate classical D_2-receptor-coupled G-protein signalling pathways. Furthermore,

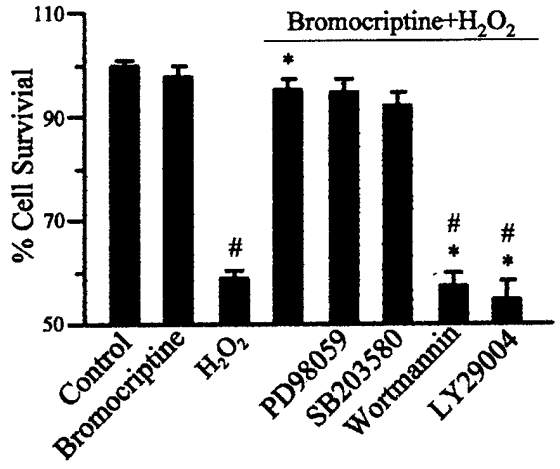

Figure 8 Elimination of DA-agonist-mediated neuroprotection by inhibition of PI 3-kinase

Low concentrations of the PI 3-kinase inhibitor wortmannin (100 nM) or LY294002 (10 μM) eliminate the protective action of bromocriptine on H_2O_2-induced apoptosis. Data plotted are from one experiment (means $\pm$ S.E.M., $n = 8$), representative of four independent experiments. #$P < 0.001$ compared with control (no treatment). *$P < 0.001$ compared with H_2O_2 alone. #*$P < 0.001$ compared with bromocriptine + H_2O_2.

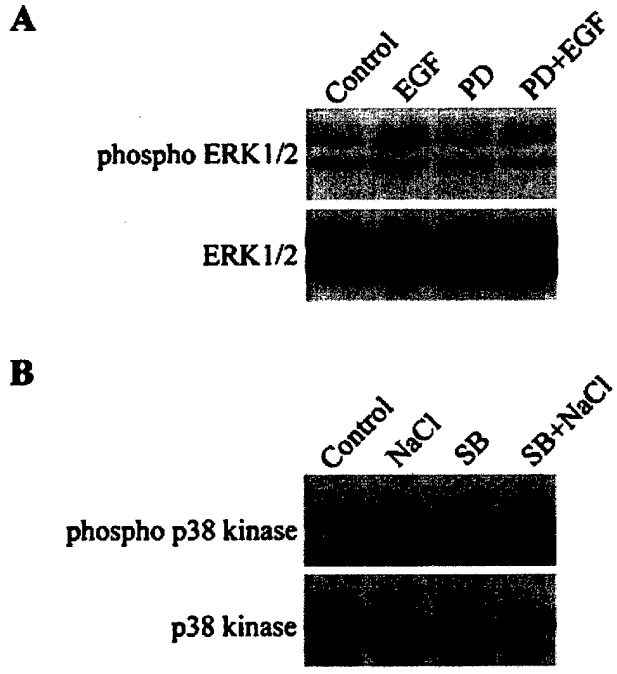

Figure 9 Representative Western immunoblots showing the effect of mitogen-activated protein kinase inhibitors on the phosphorylation of ERK and p38 kinase

(**A**) PC12-D_2R cells untreated (control) or pretreated with 50 μM PD98059 (PD) for 1 h were stimulated with epidermal growth factor (EGF) for 10 min. Western blot analysis was performed using antibodies specific for phosphorylated and total ERK1/2. (**B**) Cells were untreated (control) or pretreated with 20 μM SB203580 (SB) for 1 h and either untreated or treated with NaCl (0.4 M) for 1 h. Western blot analysis was performed using antibodies specific for phosphorylated and total p38 kinase.

we find that activation of the PI 3-kinase signalling pathway is required for anti-apoptotic activity of DA agonists in PC12-D_2R cells.

In our experiments in PC12 cells, we find no evidence for receptor-independent cytoprotective activities of DA agonists. Rather, we find that the activation of DA D_2 receptors is required for the prevention of apoptosis induced by H_2O_2 in undifferentiated PC12 cells and for protection against either H_2O_2 or trophic withdrawal in NGF-differentiated PC12 cells. This conclusion is based on the following observations: (i) no protection was observed in PC12 cells that do not express the D_2 receptor; (ii) the agonists bromocriptine, pergolide and quinpirole all protect PC12 cells from apoptosis in a concentration-dependent manner; (iii) this concentration-dependent protective effect is reversed by D_2 antagonists; and (iv) the promotion of cell survival is agonist-specific and independent of their capacity to activate G-protein coupled signalling pathway. Several previous studies have implicated activation of DA receptors in the neuroprotective effects observed with DA agonists [13,25,26]. For example, the capacity of bromocriptine to protect a mouse HT22 cell line against oxidative stress [26] and rodent dopaminergic neurons from levodopa-induced toxicity [27] is dependent on its ability to stimulate D_4 and D_2 DA receptors respectively. Thus several receptors may activate anti-apoptotic signalling.

Various studies have found that DA agonists can promote cell survival independently of receptor activation in several experimental paradigms [28–30]. Not all reports of DA agonist-mediated protection appear to rely on activation of DA receptors and other mechanisms may be applicable in other model systems. *In vitro* and *in vivo* studies have shown that DA agonists are capable of scavenging superoxide or hydroxyl radicals [30,31]. Pramipexole has been shown to up-regulate Bcl2 expression, which could provide an anti-apoptotic effect [32,33]. In addition, studies in isolated mitochondria have shown that the agonist can protect against membrane swelling induced by calcium or MPP^+, which could not be accounted for by direct receptor activation [34]. In addition, blockade of DA receptors in dopaminergic cell cultures in some studies does not prevent the protective properties of DA agonists [32,35]. Further, the enantiomers of apomorphine and pramipexole, which do not bind to DA receptors, have been reported to protect dopaminergic neurons from MPP^+, H_2O_2 or 6-hydroxydopamine toxicity [36]. These observations indicate that agonists can induce protective effects in some models independent of DA receptors [29,32,35]. These differences with our findings, in which the anti-apoptotic activity of DA agonists in PC12-D_2R cells is predominantly mediated by activation of the D_2 receptors, may result from differences in the experimental systems utilized and/or the DA agonist tested.

DA receptors belong to the rhodopsin family of heptahelical G-protein-coupled receptors. We find that the D_2-receptor-mediated increased cell survival is apparently independent of its activation of G_i/G_o heterotrimeric G-proteins. Recent studies have revealed that, in addition to heterotrimeric G-proteins, these receptors may interact with and activate a variety of signal mediators, including small G-proteins [37], Na^+/H^+-exchange factor [38], c-Src [39] and cGMP-operated Ca^{2+} channels [26]. The differences in their relative neuroprotective efficacy and their activation of G-proteins support the 'agonist signal trafficking' hypothesis that different agonists acting at the same receptor subtype can stabilize distinct receptor conformations and thereby preferentially activate subsets of the signalling pathways coupled to that receptor [40]. A recent study on D_2 receptor–G-protein interactions reported that specific agonists differed in their relative activity at promoting receptor complexing with either G_{i2} or G_o G-proteins, also supporting the formulation that specific agonists can stabilize the D_2 receptor in different conformation [20]. Our data show that specific agonists select between activation of only G_i/G_o G-protein pathways and the additional activation of a G-protein-independent

neuroprotective pathway. This distinction may be important in designing studies to determine the neuroprotective activity of DA agonists *in vivo*.

The present study demonstrates that PI 3-kinase inhibitors block the protective effect of D_2-receptor stimulation. Therefore, signal transduction via PI 3-kinase activation is necessary for this protective effect. Although PI 3-kinase is clearly important for growth-factor-mediated neuronal survival in many cell types and conditions, in other neuronal cell types and under different conditions, growth-factor-mediated activation of the ERK-signalling pathway appears to mediate survival effects [41]. We find no evidence for the involvement of ERK in D_2-receptor-mediated survival of PC12-D_2R cells from our studies using specific inhibitors. It was reported recently that D_2-receptor activation protects cortical neurons from glutamate-induced cytotoxicity by up-regulation of Bcl-2 protein expression via the PI 3-kinase cascade [42]. Therefore the effects of D_2-receptor activation on PI 3-kinase and cell survival appear to apply to a variety of cellular insults.

We find that a remarkably large effect on survival can be obtained with activation of the D_2 receptor by certain agonists. Furthermore, this improvement in survival is quite different with different agonists and does not correlate with the capacity of these agonists to activate classical G-protein signalling. These results suggest that D_2 receptors activate a trophic factor linked pro-survival signalling pathway in an agonist-specific manner. Thus it is likely that intracellular signalling by D_2-receptor stimulation can be manipulated for the development of more effective neuroprotective therapies.

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Agonist-specific Transactivation of Phosphoinositide 3-Kinase Signaling Pathway Mediated by the Dopamine D_2 Receptor*

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Bromocriptine, acting through the dopamine D_2 receptor, provides robust protection against apoptosis induced by oxidative stress in PC12-D_2R and immortalized nigral dopamine cells. We now report the characterization of the D_2 receptor signaling pathways mediating the cytoprotection. Bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either phosphoinositide (PI) 3-kinase, epidermal growth factor receptor (EGFR), or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress. Co-immunoprecipitation studies showed that the D_2 receptor forms a complex with the EGFR and c-Src that was augmented by bromocriptine, suggesting a cross-talk between these proteins in mediating the activation of Akt. EGFR repression by inhibitor or by RNA interference eliminated the activation of Akt by bromocriptine. D_2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphorylation specifically at tyrosine 845, a known substrate of Src kinase. Furthermore, Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation. Thus, the predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-kinase and Akt. We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D_2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in this experimental system. These results support the hypothesis that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in their coupling to G-proteins and to a cytoprotective c-Src/EGFR-mediated PI-3 kinase/Akt pathway.

The hallmark of Parkinson's disease (PD)[1] is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (1), causing a profound reduction in dopamine-mediated signaling (2). The prominent locomotor deficits that occur in this disease are in large part attributable to the loss of stimulation of the dopamine D_2 receptor (3), a member of the rhodopsin-like heptahelical receptor family (4). The D_2 receptor is an important target for anti-parkinsonian drugs that ameliorate the motor deficits associated with this disorder. In recent years, dopamine agonists have also been found to have neuroprotective activity in some experimental models, and the possibility that they may decrease the progression of PD has been proposed (5). However, the mechanisms underlying the agonist-mediated neuroprotection reported in experimental models are poorly understood, and the potential for dopamine agonists to alter the clinical course of this disease remains an area of controversy (6).

Many heptahelical receptors couple to multiple signal transduction pathways, including various heterotrimeric G-protein-second messenger pathways and growth factor receptor-protein kinase cascades (7). The signal for activation of the proximal mediators of signaling such as heterotrimeric G-proteins, receptor kinases, or other protein partners, is an alteration in the receptor's conformation that occurs following complexing with agonist. Studies in several heptahelical receptors suggest that these proteins exist in multiple, functionally significant conformations that may differ in their relative activation of different signaling pathways (8–12). Studies with several receptors, including the dopamine D_2 receptor, suggest that agonists acting at the same receptor select among different active receptor conformations and determine the relative levels of activation of downstream signaling pathways, a hypothesis called agonist-directed signal trafficking (13–16).

We had previously investigated the role of the D_2 receptor expressed in the PC12 cell line (PC12-D_2R) in modulating the induction of apoptotic cell loss caused by hydrogen peroxide-induced (H_2O_2) oxidative stress (17). Although the mechanism of neuronal loss in PD is not known, many studies have implicated oxidative stress (reviewed in Refs. 18 and 19). Oxidation of dopamine by auto-oxidation and monoamine oxidase produces reactive oxygen species, including H_2O_2. H_2O_2 reacts with ferrous (Fe^{+}_{2}) iron to produce hydroxyl radicals, which can damage proteins, nucleic acids, and membrane phospholipids, and induce apoptosis (20). Some animal model and human PD postmortem studies provide evidence that the degeneration of DA neurons occurs via apoptosis (21, 22). We found that activation of the D_2 receptor in the PC12-D_2R line caused a robust, concentration-dependent increase in cell survival during oxidative stress that required activation of phosphoinositide 3-kinase (PI 3-kinase). Among the agonists studied, we found significant discrepancies in the capacity of individual agonists to

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[1] The abbreviations used are: PD, Parkinson's disease; PI, phosphoinositide; Akt, protein kinase B; PH, pleckstrin homology; GFP, green fluorescent protein; EGFR, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; PTX, pertussis toxin; ECL, enhanced chemiluminescence lighting; PIP_3, phosphatidylinositol 3,4,5-triphosphate; PDGF, platelet-derived growth factor; siRNA, small interfering RNA; GPCR, G-protein-coupled receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; PP2, 4-amino-5(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4-*d*]pyrimidine.

mediate anti-apoptosis and to stimulate G-protein activation, assayed via [^{35}S]GTPγS binding (17).

To elucidate the mechanisms underlying agonist-specific modulation of cell survival, we have now investigated the anti-apoptotic signaling pathway activated by the D_2 receptor. We find that D_2 receptor-mediated protection against oxidative stress involves a novel c-Src-dependent transactivation of the epidermal growth factor receptor (EGFR) that activates PI 3-kinase/protein kinase B (Akt) and that agonists differ in their capacity to activate this pathway.

EXPERIMENTAL PROCEDURES

Chemicals—(+)-Bromocriptine methanesulfonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), H_2O_2, nerve growth factor (NGF) and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St. Louis, MO). Pramipexole was a gift from Amersham Biosciences (Kalamazoo, MI). LipofectAMINE, DMEM, and fetal calf serum were obtained from Invitrogen (Gaithersburg, MD). AG1478, AG1296, k252a, PP2, LY294002, and wortmannin were obtained from Calbiochem (La Jolla, CA). Epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies specific to phospho-Akt, Akt, phospho-tyrosine EGFR, EGFR were from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src and c-Src antibodies were from BIOSOURCE International Inc. (Camarillo, CA). Mouse monoclonal antibodies to dopamine D_2 receptor, phospho-tyrosine (PY20), and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Viability Analysis—The development and characterization of the PC12-D_2R cell line, which is stably transfected with the human D_{2L} receptors, were previously described (17). The cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, and 500 μg/ml G418 in a humidified atmosphere containing 5% CO_2 at 37 °C. For differentiation, PC12-D_2R cells were plated onto collagen-coated plates in DMEM containing 10% horse serum and 5% fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in DMEM supplemented with 1% fetal bovine serum and 100 ng/ml NGF for 10–14 days. Nigral dopamine cell line SN4741 (generous gift from Dr. J. H. Son, Columbia University, New York, NY) was cultured as described previously (23). Cell viability was measured by the MTT method 24 h after various treatments as described (17).

Transfections and DNA Constructs—For live cell fluorescence microscopy, PC12-D_2R cells (1×10^5) were plated into 60-mm culture dishes and incubated in the media for 24 h. The media was replaced with serum-free DMEM, and a mixture containing 5 μg of the plasmid DNA encoding the pleckstrin homology domain of Akt protein kinase (1–167) tagged with green fluorescent protein (PH-Akt-GFP) (24) (kindly provided by Dr. T. Balla, National Institutes of Health, Bethesda, MD) and 30 μl of LipofectAMINE reagent were gently added to each plate and incubated for 3 h at 37 °C at 5% CO_2. The DNA-containing medium was replaced with fresh DMEM-containing serum. When co-transfection of c-Src (wild type) or dominant negative c-Src (K295R/Y527F) (both were generous gifts from Dr. J. Burgge, Harvard Medical School, Boston, MA) with PH-Akt-GFP was carried out, the DNA concentration used was 1:1. Green fluorescent protein (GFP) plasmid was from Clontech. SN4741 cells were co-transfected with D_{2L} and PH-Akt-GFP or GFP by calcium phosphate method (25).

Epifluorescence Imaging—Microscopy of live cells transfected to express PH-Akt-GFP was performed on the Olympus (BX65) upright fluorescent microscope using a water immersion objective lens (×40) fitted with a heated stage and an objective lens heater. Images were collected at 2-min intervals after the addition of the drugs and processed using Adobe Photoshop (5.5).

Immunoblotting and Immunoprecipitation—PC12-D_2R cells (1×10^6 cells/100-mm plate) were grown for 24 h, and following respective treatments, the cells were washed twice with ice-cold phosphate-buffered saline and lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal C630, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, and mixture of protease inhibitors (Roche Applied Science, GmbH) at 4 °C for 20 min. After centrifugation at 14,000 × *g* for 20 min at 4 °C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was conducted using ECL system according to the manufacturer's recommendations. The blots were then stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and re-probed with respective antibodies.

For immunoprecipitation, the protein extract was incubated sequentially (2 h for each incubation at 4 °C) with anti-D_2 receptor antibody and protein A/G-agarose with gentle agitation. Immunoprecipitates were washed three times with lysis buffer, boiled for 5 min in 3× Laemmli sample buffer, and processed for Western blotting using EGFR or c-Src antibody. The EGFR blots were stripped and reprobed with anti-phospho tyrosine (PY20) antibody.

RNA Interference—Custom SMARTpool plus small interfering RNA (siRNA) to target rat EGFR (catalog no. M-004710-00) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (50 pmol) was co-transfected with PH-Akt-GFP (2 μg) into PC12-D_2R cells using transit-TKO and -neural transfection reagents (Mirus, Madison, WI) according to manufacturer's protocol. For immunofluorescence, 24 h after transfection, cells were serum-starved for 1 h and were treated with bromocriptine for 10 min. The cells were fixed with ice-cold methanol and immunostained for EGFR and visualized using CY3-conjugated secondary antibody. A nonspecific RNA duplex (Dharmacon, catalog no. D-001206-09-05) was used in control experiments.

RESULTS

Neuroprotection by D_2 Receptor Activation Involves PI 3-Kinase/Akt Signaling Cascade—We have previously reported that the increased cell survival in PC12-D_2R cells mediated by D_2 receptor activation was completely abolished by inhibitors of PI 3-kinase, suggesting that the D_2 receptor may be altering cell survival by activating PI 3-kinase (17). We therefore studied whether PI 3-kinase/Akt signaling was modulated by the D_2 receptor when complexed with an agonist that prevents apoptosis in these cells. Activation of PI 3-kinase generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) and thereby stimulates anti-apoptotic proteins (26). The downstream PI 3-kinase target, protein kinase B (Akt), has been reported to be important in mediating survival in many cell types (27). Akt is activated by phosphorylation at Thr^{308} in the catalytic loop and Ser^{473} in the C-terminal domain (28, 29).

We first determined whether the anti-apoptotic dopamine agonist bromocriptine induced phosphorylation of Ser^{473} of endogenous Akt in PC12-D_2R cells. As shown in Fig. 1*A*, Akt phosphorylation was increased 15 min after exposure to bromocriptine. In some cell lines, H_2O_2 has been reported to activate Akt (30, 31). However, we found that in PC12-D_2R cells H_2O_2 alone had no effect on the phosphorylation of Akt (Fig. 1*A*). Akt phosphorylation occurs after it is recruited to the plasma membrane through an interaction of its N-terminal pleckstrin homology (PH) domain with PIP_3 (32), thereby bringing the enzyme into the proximity of additional PIP_3-dependent and -independent protein kinases (33). We studied the redistribution of Akt by D_2 receptor signaling using a PH-Akt-GFP fusion protein (24). The localization of PH-Akt-GFP in quiescent PC12-D_2R cells was indistinguishable from that of transfected GFP alone. Receptor activation by bromocriptine, however, caused a rapid (<5 min) translocation of the PH-Akt-GFP to ruffled membrane regions (Fig. 1*B*, *top panels*). No response to bromocriptine was observed in control PC12-D_2R cells expressing GFP alone (Fig. 1*B*, *middle panels*) or in the parent PC12 cells, which lack the D_2 receptor, expressing PH-Akt-GFP (Fig. 1*B*, *bottom panels*). We also tested whether this pathway was active in cells exposed to oxidative stress. As shown in Fig. 1*C*, the bromocriptine-induced phosphorylation of Akt and translocation of PH-Akt-GFP (Fig. 1*D*) were unaffected in the presence of H_2O_2. Thus, D_2 receptor stimulation by bromocriptine caused translocation and phosphorylation of Akt in PC12-D_2R cells during oxidative stress.

D_2 Receptor Activation of Akt Is PTX-insensitive and PI 3-Kinase-dependent—The D_2 receptor is a member of the rhodop-

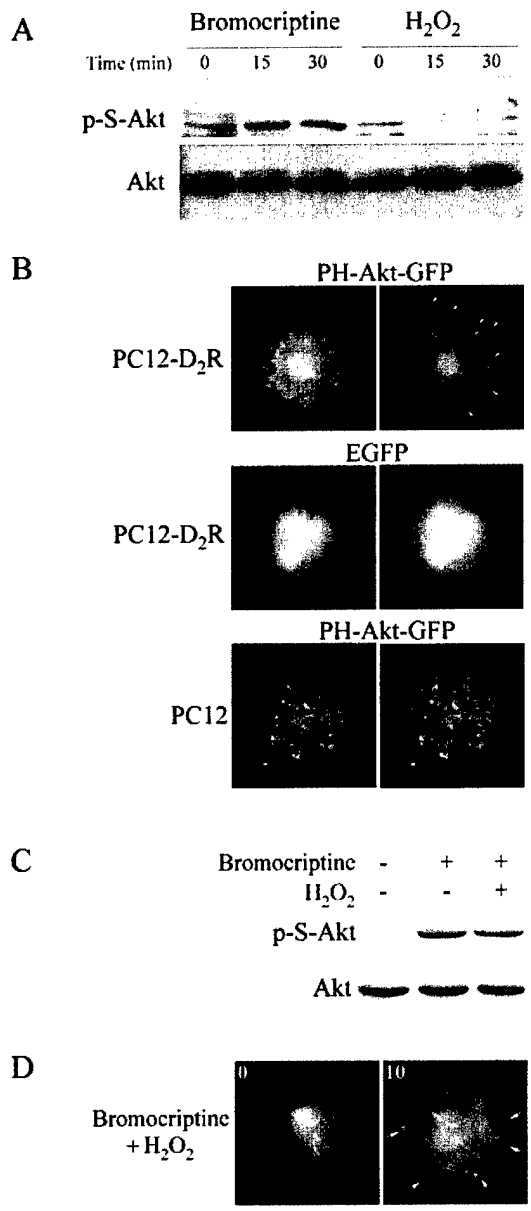

FIG. 1. **D_2 receptor stimulation activates Akt in PC12-D_2R cells.** *A*, phosphorylation of Akt by the D_2 receptor agonist bromocriptine. PC12-D_2R cells were stimulated with 1 μM bromocriptine or H_2O_2 (200 μM) for the periods of time indicated. After stimulation, cells lysates were prepared and analyzed by Western blotting with anti-phospho-Akt antibody or with anti-Akt antibody. *B*, translocation of PH-Akt-GFP by D_2 receptor stimulation. PC12-D_2R cells expressing PH-Akt-GFP (48 h after transfection) were stimulated with the D_2 agonist bromocriptine (100 nM), and translocation of PH-Akt-GFP was determined by live cell imaging. Each pair of *left* (0 min) and *right* (10 min) *panels* represents the images captured from the same living cell. Bromocriptine stimulates translocation of PH-Akt-GFP reporter to the membrane within 10 min (*top panels*). The *arrows* indicate localized areas of PH-Akt-GFP translocation following addition of bromocriptine. Bromocriptine (100 nM) had no effect on the translocation of or EGFP expressed in PC12-D_2R cells (*middle panels*) or PH-Akt-GFP expressed in native PC12 cells, which lack D_2 receptors (*bottom panels*). Panels shown are from one of eight independent experiments. *C*, oxidative stress had no effect on the phosphorylation or translocation of Akt in the presence of bromocriptine. Western blot of phospho-Akt. *Lane 1*, control; *lane 2*, bromocriptine (1 μM); *lane 3*, bromocriptine plus H_2O_2 (200 μM). All the incubations were carried out for 15 min. *D*, bromocriptine-induced translocation of PH-Akt-GFP was unaffected in presence of H_2O_2. Live cell imaging was carried out following addition of H_2O_2 (200 μM) and bromocriptine (100 nM) into the medium. Each pair of *left* (0 min) and *right* (10 min) *panels* represents the images captured from the same living cell. *Arrows* indicate localized areas of PH-Akt-GFP translocation following addition of bromocriptine. The *panels* shown are from one of six independent experiments.

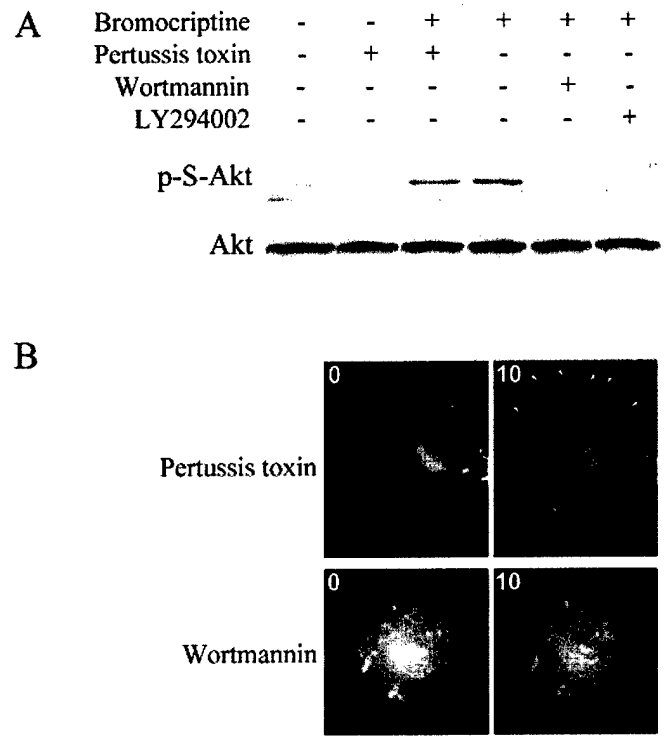

FIG. 2. **Activation of PI 3-kinase/Akt signaling cascade by D_2 receptor is independent of PTX-sensitive G-protein-coupled pathway.** *A*, Western blot showing the phosphorylation of Akt by bromocriptine in presence of PTX, but no stimulation of Akt phosphorylation in presence of PI 3-kinase inhibitors LY 294002 or wortmannin. PC12-D_2R cells were either left untreated or pretreated with PTX (100 ng/ml; 16 h), wortmannin (100 nM), or LY294002 (20 μM) for 1 h and then stimulated with 1 μM bromocriptine for 15 min. *B*, effect of PTX or PI 3-kinase inhibitors on translocation of PH-Akt-GFP by bromocriptine (100 nM). PTX (100 ng/ml, 16 h) pretreatment had no effect on the bromocriptine-induced redistribution of PH-Akt-GFP (*top panels*). Pretreatment of cells with PI 3-kinase inhibitor wortmannin (100 nM, 1 h) completely inhibited the bromocriptine-induced redistribution of PH-Akt-GFP (*bottom panels*). *Arrows* indicate localized areas of PH-Akt-GFP following addition of bromocriptine. *Panels* shown are from one of six independent experiments.

sin-like heptahelical receptor family, whose classic signaling pathway involves the activation of the G_i/G_o subtype heterotrimeric G-proteins, which can be inactivated by PTX (34). To examine the role of G_i/G_o coupling in activation of PI 3-kinase/Akt, we determined the effects of PTX on these responses. Pretreatment of PC12-D_2R cells with 100 ng/ml PTX (16 h) eliminated bromocriptine-stimulated [^{35}S]GTPγS binding (data not shown). In contrast, the D_2 receptor-mediated phosphorylation of Akt was unaffected by PTX (Fig. 2*A*, compare *lanes 3* and *4*). To assess the role of PI 3-kinase in the activation of Akt induced by D_2 receptors, PC12-D_2R cells were pretreated with the inhibitor wortmannin at 100 nM, a concentration that selectively blocks PI 3-kinase (35). The cultures were then exposed to bromocriptine. Wortmannin completely prevented the phosphorylation of Akt that is inducible by D_2 receptor activation (Fig. 2*A*, compare *lanes 4* and *5*). Similar results were also obtained with LY294002 (20 μM), another commonly used but less potent inhibitor of PI 3-kinase (35) (Fig. 2*A*, compare *lanes 4* and *6*). Translocation of PH-Akt-GFP by bromocriptine was also inhibited by pretreatment with the PI 3-kinase inhibitor wortmannin, whereas it was unaffected by PTX pretreatment (Fig. 2*B*). Similar results were obtained with LY294002 (data not shown). These results indicate that the D_2R-mediated activation of Akt occurs through PI 3-kinase by a mechanism independent of G_i/G_o class heterotrimeric G-proteins.

D_2 Receptor Activates PI 3-kinase/Akt Pathway in Nigral Dopamine Cells—To explore whether the D_2 receptor coupling

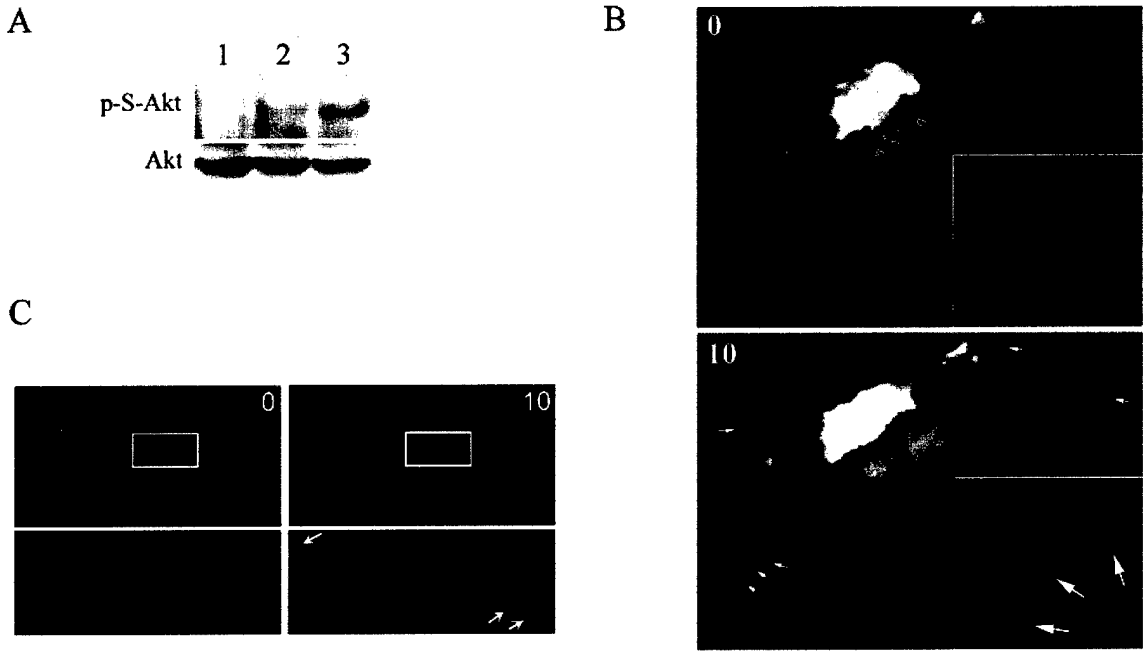

FIG. 3. **D_2 receptor activates Akt in immortalized nigral DA cell line (SN4741).** *A*, phosphorylation of endogenous Akt by bromocriptine in SN DA cells (*top*) and total Akt (*bottom*). *Lane 1*, control; *lanes 2* and *3*, cells incubated (15 min) with 100 nM and 1 μM bromocriptine, respectively. *B*, SN4741 cells co-expressing human D_2 receptor and PH-Akt-GFP was stimulated with bromocriptine (100 nM) and live cell imaging was carried out described under "Experimental Procedures." Bromocriptine stimulated the redistribution of PH-Akt-GFP to discrete areas as indicated by *arrows* in the cells within 10 min (*bottom panel*). *Panels* shown are from one of six independent experiments. *C*, in differentiated PC12-D_2R cells, bromocriptine (100 nM) stimulated redistribution of PH-Akt-GFP to discrete locations in cell processes as indicated by the *arrows*. *Panels* shown are from one of six independent experiments.

to PI 3-kinase/Akt observed in PC12-D_2R cells was present when the D_2 receptor was expressed in a different cellular context, we studied this signaling pathway in the mouse immortalized nigral dopamine cell line SN4741, which expresses tyrosine hydroxylase, the dopamine transporter, and D_2 autoreceptors (23). Activation of D_2 receptors by bromocriptine in these cells was found to induce phosphorylation of endogenous Akt (Fig. 3*A*). When the cells were transfected with the PH-Akt-GFP construct, bromocriptine induced redistribution of this reporter (data not shown). The capacity of the activated D_2 receptor to induce Akt redistribution in this model was enhanced in cells co-transfected with D_2 receptor and PH-Akt-GFP. Bromocriptine caused a translocation of the PH-Akt-GFP protein into discrete regions of the SN4741 cells (Fig. 3*B*) that was similar to the response observed in differentiated PC12-D_2R cells (Fig. 3*C*). The bromocriptine-stimulated translocation of PH-Akt-GFP in SN4741 cells was eliminated by pretreatment with the PI 3-kinase inhibitor LY290042 (data not shown). Control experiments in which cells were transfected with the D_2 receptor and GFP showed no change in the distribution of fluorescence in response to bromocriptine. These results suggest that the D_2 receptor can couple to the PI 3-kinase/Akt signaling pathway in dopaminergic neurons.

D_2 Receptor-mediated Neuroprotection and Activation of PI3-Kinase/Akt Involves EGFR Transactivation—We previously reported that bromocriptine showed significant PI 3-kinase-dependent anti-apoptotic activity in PC12-D_2R cells and have demonstrated, as described above, that bromocriptine also induced Akt phosphorylation and translocation. We next attempted to delineate the signal mediators connecting the D_2 receptor to PI 3-kinase. It has been reported that the PI 3-kinase/Akt pathway in PC12 cells can be activated by receptor tyrosine kinases (36). The effectiveness of several receptor tyrosine kinase inhibitors on bromocriptine-mediated neuroprotection was evaluated. H_2O_2 exposure caused significant loss of PC12-D_2R cell viability at 24 h, as determined using the MTT metabolism assay, and this cell loss was nearly completely reversed by the D_2 receptor agonist bromocriptine (Fig. 4*A*), consistent with our previous results (17). The effects of various growth factor receptor inhibitors on the capacity of bromocriptine to protect cells against cell death due to H_2O_2 exposure were studied. AG1296 (200 nM), AG1478 (200 nM), and k252a (50 nM) in the presence of H_2O_2 and the presence or absence of bromocriptine (100 nM) for 24 h were evaluated. As shown in Fig. 4*A*, AG1478, a specific inhibitor of EGFR intrinsic tyrosine kinase activity (37), completely abolished the neuroprotection provided by bromocriptine exposure, an effect similar to that observed with inhibition of PI 3-kinase (17). In contrast, inhibition of platelet-derived growth factor (PDGF) receptors by AG1296 or NGF receptor by k252a had no effect on D_2 receptor-mediated cell survival.

We next investigated the role of the EGFR in mediating the signaling from the D_2 receptor to Akt. Activation of the EGFR by EGF caused a rapid phosphorylation of Akt and a translocation of PH-Akt-GFP in PC12-D_2R cells, similar to the response observed with bromocriptine (Fig. 4*B*). The involvement of EGFR transactivation in D_2 receptor stimulation of Akt phosphorylation and translocation was supported by finding a complete inhibition of these responses after pretreatment with AG1478 (Fig. 4*C*). These results suggest that PI 3-kinase/Akt is one of the downstream effectors of the EGFR and that the D_2 receptor activates PI 3-kinase/Akt via transactivation of the EGFR in PC12-D_2R cells.

To confirm the role of the EGFR in the activation of PI-3 kinase/Akt by D_2 receptors, we reduced the levels of EGFR expression in PC12-D_2R cells using RNA interference. After transfection with EGFR-specific or control small interfering RNA (siRNA), cultures were assessed for EGFR protein expression by immunofluorescence. As shown in Fig. 5, EGFR was substantially repressed by 24 h post-transfection in ~60–70% of the cells. The involvement of EGFR transactivation in D_2 receptor-stimulation of Akt was studied in PC12-D_2R cells co-

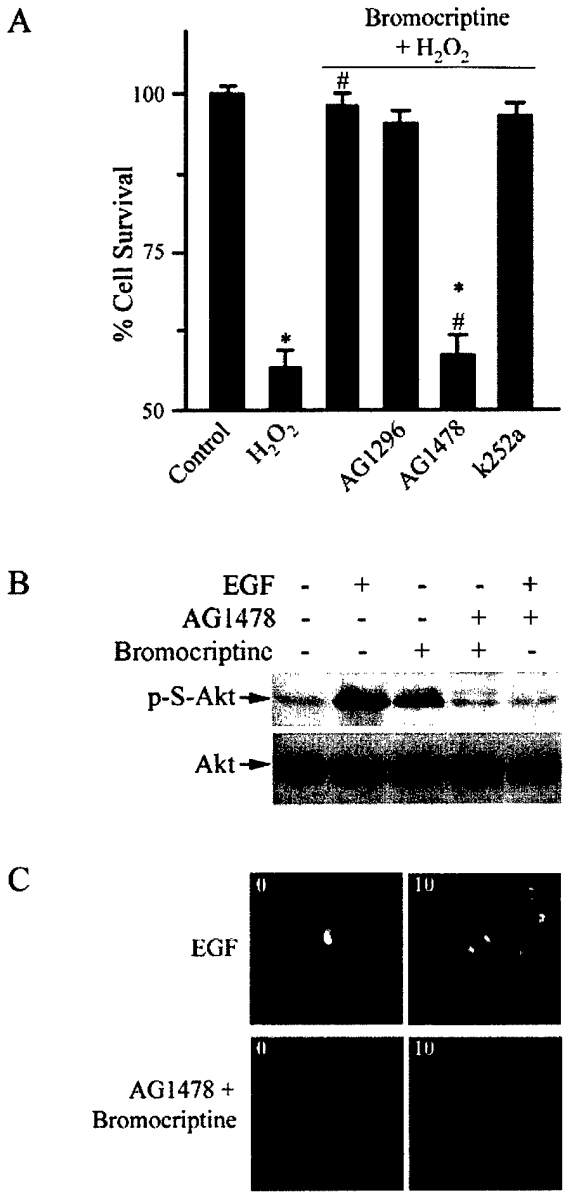

FIG. 4. *A*, elimination of dopamine agonist-mediated neuroprotection by inhibition of EGFR. The EGFR inhibitor AG1478 (200 nM) eliminates the protective action of bromocriptine (100 nM) on H_2O_2-induced apoptosis. Data are plotted from one experiment (mean ± S.E., $n = 8$), representative of four independent experiments. *, $p < 0.001$ compared with control (no treatment). #, $p < 0.001$ compared with H_2O_2. #*, $p < 0.001$ compared with H_2O_2 plus bromocriptine. Inhibition of PDGF receptor by AG1296 (200 nM) or NGF receptors by k252a (50 nM) had no effect on the protection mediated by bromocriptine. *B*, effect of EGFR inhibitor on D_2 receptor-mediated phosphorylation of Akt in PC12-D_2R cells. Cells were either left untreated or pretreated with EGFR inhibitor, AG1478 (200 nM), for 30 min and then stimulated with 1 μM bromocriptine for 15 min or EGF (100 ng/ml) for 10 min. After stimulation, cells were lysed and lysates were analyzed by Western blotting with either anti-phosphorylated Akt or anti-Akt antibody. *C*, effect of EGFR inhibitor on D_2 receptor-mediated translocation of Akt in PC12-D_2R cells. EGFR inhibition impaired PH-Akt-GFP translocation following D_2 receptor stimulation. EGF (100 ng/ml) translocates PH-Akt-GFP to discrete areas (*top panels*) and pretreatment with AG1478 (200 nM, 30 min) inhibit PH-Akt-GFP translocation by bromocriptine (*bottom panels*).

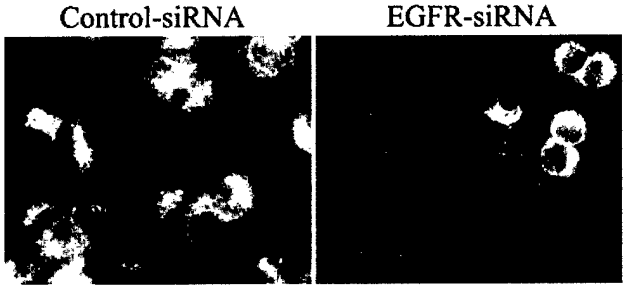

FIG. 5. **Gene silencing of EGFR by RNA interference.** PC12-D_2R cells were transfected with either control siRNA or EGFR siRNA as described under "Experimental Procedures." The cells were immunostained with anti-EGFR antibody 24 h after transfection. The EGFR expression was assessed by immunofluorescence microscopy. Note the substantial loss of EGFR immunoreactivity in the majority of EGFR-siRNA-transfected cells.

transfected with PH-Akt-GFP and EGFR siRNA or control siRNA. 24 h after transfection the cells were serum-starved for 1 h, stimulated with bromocriptine, and assessed for the expression of EGFR and the translocation of PH-Akt-GFP. In control siRNA-transfected cells, PH-Akt-GFP translocation was similar to that observed in cells not transfected with siRNA (Fig. 6*A* and data not shown). However, EGFR repression by siRNA completely blocked the translocation of PH-Akt-GFP by bromocriptine (Fig. 6*B*). We conclude that EGFR is essential for the translocation of PH-Akt-GFP following D_2 receptor stimulation.

To clarify the mechanisms through which the D_2 receptor transactivates the EGFR, we examined the association between these two membrane proteins. After exposure of cells to vehicle or bromocriptine and preparation of cell extracts, we used a specific anti-D_2 receptor monoclonal antibody (Fig. 7*A*) to perform immunoprecipitations followed by immunoblotting for the EGFR (Fig. 7*B*). These studies showed that the EGFR co-immunoprecipitated with the D_2 receptor and the association between these two proteins was augmented in the presence of bromocriptine. Furthermore, the EGFR that complexed with the D_2 receptor in the presence of bromocriptine showed an increase in Tyr phosphorylation. These data indicate that the D_2 receptor and EGFR form a complex and that their association is augmented by bromocriptine. We also examined this complex for the presence of c-Src, which was detected in extracts immunoprecipitated by the D_2 receptor antibody. The presence of c-Src in this complex was also enhanced by exposure of the cells to bromocriptine (Fig. 7*C*).

The sites of EGFR Tyr phosphorylation induced by bromocriptine were studied using site-specific anti-phosphotyrosine antibodies. We analyzed tyrosine phosphorylation of the EGFR at residues 992 and 1068, which are EGFR autophosphorylation sites (38) and at residue 845 (Tyr^{845}), a known Src phosphorylation site (39). As shown in Fig. 8, incubation of PC12-D_2R cells with EGF increased the phosphorylation of tyrosine residues 845, 992, and 1068, whereas bromocriptine only enhanced phosphorylation of Tyr^{845}. The bromocriptine-mediated phosphorylation of Tyr^{845} was inhibited by pretreatment with EGFR inhibitor (Fig. 8*C*).

EGFR Transactivation Is c-Src-dependent—The finding that bromocriptine enhanced the association of the D_2 receptor with c-Src and induced phosphorylation of Tyr^{845}, a Src-dependent phosphorylation site of the EGFR (39), led us to study further the role of c-Src in this signaling. Src family kinases have been implicated in the phosphorylation of the EGFR and of PI 3-kinase (39–41). We examined the phosphorylation of Tyr^{418} in c-Src, which is an autophosphorylation site required for kinase activity of c-Src (42). Cells were exposed to bromocriptine (100 nM) for periods up to 30 min. To determine whether c-Src was activated by D_2 receptor stimulation, we performed immunoblotting using an antibody specific for c-Src phospho-Tyr^{418}. Stimulation of the D_2 receptor by bromocriptine caused c-Src to be phosphorylated at Tyr^{418} (Fig. 9*A*).

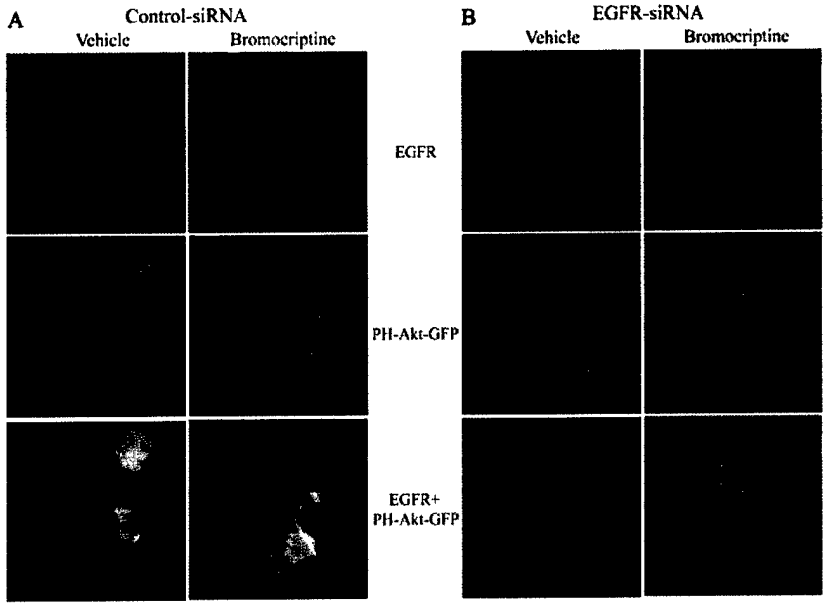

FIG. 6. **Gene silencing of EGFR inhibits its D_2 receptor signaling to Akt.** EGFR siRNA inhibited bromocriptine-induced PH-Akt-GFP translocation. PC12-D_2R cells were transfected with EGFR siRNA or control siRNA together with PH-Akt-GFP plasmid DNA. The cells were treated with bromocriptine (100 nM) for 10 min, and PH-Akt-GFP translocation and EGFR expression were examined in these cells. Each set of *three vertical panels* represents the same field. EGFR immunofluorescence is indicated in *red* (*top panels*). PH-Akt-GFP signal is indicated in *green* (*middle panels*). The *bottom panels* are overlays of both EGFR and PH-Akt-GFP signals. *A*, bromocriptine induced a characteristic ring-like margination of PH-Akt-GFP and concentration of PH-Akt-GFP into membrane processes in cells transfected with control siRNA. *B*, suppression of EGFR expression by EGFR siRNA eliminated the redistribution of PH-Akt-GFP by bromocriptine. Note that these images are from fixed cells and are not identical in appearance to the live cell images shown in Fig. 1. Data shown are representative of three independent experiments.

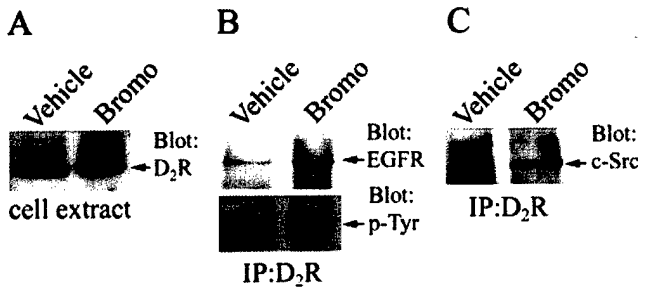

FIG. 7. **Bromocriptine induced the association of D_2 receptor with EGFR and c-Src.** PC12-D_2R cells were treated with either vehicle or 1 μM bromocriptine for 10 min, and whole cell extracts were prepared. *A*, equal amount of cell extract, prior to immunoprecipitation, probed with the anti-D_2 receptor monoclonal antibody to establish specificity of the antibody. *B*, cell extracts were immunoprecipitated with monoclonal anti-D_2 receptor antibody and immunoblotted with antibodies to EGFR (*upper panel*). Stripping and reprobing with monoclonal anti-phosphotyrosine antibody revealed EGFR tyrosine phosphorylation associated with bromocriptine stimulated D_2 receptor (*lower panel*). *C*, immunoblot of D_2 receptor immunoprecipitate stained with anti-c-Src antibody. Note that activation of the D_2 receptor with bromocriptine increased the association of the receptor with both the EGFR and c-Src. All blots shown are representative of two to three independent experiments.

We then examined the effects of the Src family tyrosine kinase inhibitor PP2 on D_2 receptor-mediated Akt phosphorylation and cell survival. PP2 completely inhibited both the capacity of bromocriptine to induce phosphorylation of Akt (Fig. 9*B*) and to mediate cell survival in the presence of oxidative stress (data not shown). PP2 also prevented the ability of bromocriptine treatment to induce the phosphorylation of c-Src-Tyr^{418} and EGFR-Tyr^{845} (Fig. 8*C*). However, inhibition of the EGFR by AG1478 did not affect the capacity of bromocriptine to induce phosphorylation of c-Src (Fig. 9*C*), suggesting that the EGFR is downstream of c-Src in D_2 receptor signaling. The role of c-Src in the D_2 receptor signaling was further evaluated using a dominant negative c-Src construct. When co-expressed with PH-Akt-GFP, the dominant negative c-Src kinase (k295R/Y527F) completely abolished translocation of PH-Akt-GFP in response to bromocriptine (Fig. 9*D*). Thus both pharmacological inhibition and dominant negative studies indicate that c-Src activation is required for signaling from the D_2 receptor through the EGFR to the neuroprotective PI 3-kinase/Akt pathway.

Translocation and Phosphorylation of Akt by D_2 Receptor Stimulation Are Agonist-specific—We had previously found that D_2 receptor agonists varied greatly in their capacity to mediate increased survival of PC12-D_2R cells and that their protective efficacy showed no correlation with G-protein activation, as assayed by GTPγS binding. In particular, the efficacy of the agonists bromocriptine and pramipexole for GTPγS binding were indistinguishable, whereas pramipexole was essentially devoid of neuroprotective activity in the PC12-D_2R model. Having implicated the PI 3-kinase/Akt signaling pathway in the neuroprotection mediated by the D_2 receptor when complexed with bromocriptine, we were interested in determining the effects of pramipexole on this pathway. As shown in Fig. 10, pramipexole failed both to induce translocation of PH-Akt-GFP and phosphorylation of Akt in PC12-D_2R cells. These results suggest that specific agonists that interact with the dopamine D_2 receptor differ markedly in their relative activation of classic and growth factor signaling pathways when complexed with the D_2 receptor (see "Discussion").

DISCUSSION

We have delineated a D_2 receptor-activated signaling pathway that mediates neuroprotection by specific D_2 agonists in dopaminergic cell lines. Bromocriptine stimulates the PI 3-kinase/Akt pathway through a PTX-insensitive mechanism involving c-Src and transactivation of the EGFR. Our results suggest that the relative activation of classic G-protein and growth factor signaling pathways by the D_2 receptor is agonist-specific.

Because bromocriptine can induce the activation of the PI 3-kinase/Akt pathway and in many circumstances the modulation of Akt signaling normally occurs via growth factor stimulation, we sought to determine if the effects of bromocriptine on PC12-D_2R cells were mediated through a growth factor receptor. Here, we report that the bromocriptine induced the activation of Akt within minutes and this activation required the EGFR. We show that EGFR-specific tyrosine kinase inhibitor completely block bromocriptine-induced activation of Akt. Furthermore, EGFR repression by siRNA also inhibited the translocation of PH-Akt-GFP by bromocriptine. Inhibitor and dominant negative Src studies show that the activation of the EGFR by the D_2 receptor involves Src. Co-immunoprecipitation studies show that the D_2 receptor complexes with the EGFR and with c-Src and that this association is enhanced by D_2

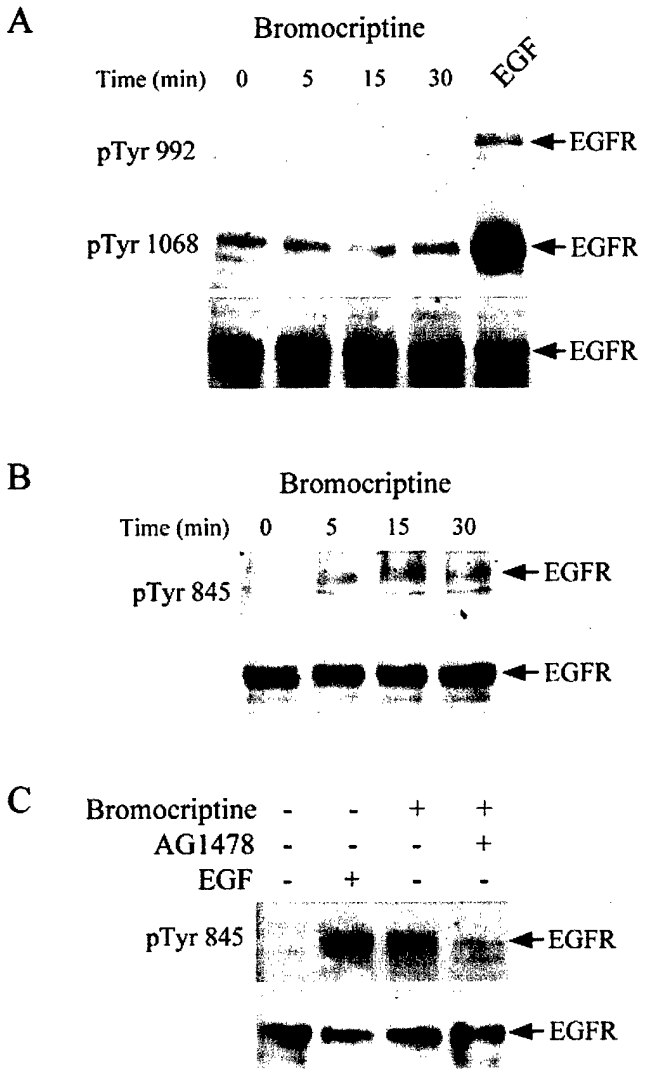

FIG. 8. **Tyrosine phosphorylation of residue 845 of the EGFR by bromocriptine.** PC12-D_2R cells were incubated with 1 μM bromocriptine for the indicated time points and EGF for 10 min. The cells were then lysed, and the lysates were resolved by SDS-gel electrophoresis on 7.5% gel and immunoblotted. Tyrosine phosphorylation was detected using site-specific tyrosine antibodies to EGFR from total cell lysates using the indicated anti-phosphotyrosine antibodies. EGFR levels were detected using anti-EGFR antibody. *A*, bromocriptine had no effect on the phosphorylation of EGFR Tyr^{992} and Tyr^{1068}. *B*, bromocriptine-induced phosphorylation of EGFR Tyr^{845}. *C*, phosphorylation of EGFR Tyr^{845} by bromocriptine was inhibited by AG1478 pre-treatment (200 nM, 30 min).

receptor activation. The neuroprotective D_2 receptor signaling pathway we have characterized is summarized in Fig. 11.

Our data indicate that stimulation of c-Src/EGFR family kinases are required for Akt activation in response to bromocriptine in PC12-D_2R cells. Src family kinases have been implicated in GPCR-induced EGFR tyrosine phosphorylation, and GPCRs can induce association of Src with the EGFR (43–45). In other studies, GPCR-induced EGFR tyrosine phosphorylation was found to be Src-independent (46, 47). We found that bromocriptine caused activation of the EGFR, and inhibition of Src kinases had a significant effect on bromocriptine-induced EGFR tyrosine phosphorylation, implicating Src family kinases in bromocriptine-induced EGFR tyrosine phosphorylation. Src kinases can be activated by several heptahelical receptors (43, 48, 49) as well as by growth factor receptor stimulation (50), including the EGFR (39, 43, 51). c-Src has been reported to influence EGFR activity by mediating phosphorylation of Tyr^{845}, a consensus Src phosphorylation site in the EGFR (39). Inhibition of either Src or the EGFR impaired the ability of bromocriptine to cause activation of Akt in PC12-D_2R cells, indicating that activation of both proteins are required for this signaling. Inhibition of Src kinase also inhibited EGFR phosphorylation at Tyr^{845}, whereas inhibition of the EGFR did not prevent phosphorylation of c-Src. These results suggest that c-Src is upstream of both the EGFR and Akt. Moreover, inhibiting either c-Src or the EGFR completely abolished the capacity of bromocriptine to increase cell survival during oxidative stress. Although Akt or upstream kinases have been reported to be a substrate for c-Src phosphorylation (52, 53), in PC12-D_2R cells both c-Src and EGFR phosphorylation were required for Akt activation. Therefore we propose that, in dopaminergic neurons, the D_2 receptor transactivates the EGFR through c-Src, which in turn activates the cytoprotective PI 3-kinase/Akt pathway.

Several mechanisms have been reported for heptahelical receptor activation of Src kinase. The β3 adrenergic receptor interacts with c-Src directly via Pro-rich domains in the receptor (49). Src activation by the β2 adrenergic receptor requires arrestin (48). The D_3 receptor has been found to contain noncanonical SH3 ligands (54). Putative SH3 domains are also present in the D_2 receptor, which might potentially mediate an interaction with Src. Using co-immunoprecipitation studies, we have demonstrated that the D_2 receptor and the EGFR form a complex that includes c-Src. Our inhibitor and dominant negative Src data further support the involvement of c-Src in activation of the EGFR. Whether the activation of c-Src by the D_2 receptor occurs directly or requires additional adaptor proteins remains to be determined.

Heptahelical receptors, including D_2-class receptors, have been reported to induce activation of growth factor receptor-coupled pathways or PI 3-kinase (49, 55–64). The cellular background in which a receptor is expressed may be important in determining its signaling potential. Platelet-derived growth factor receptor (PDGF) transactivation by the D_2 and D_4 receptors expressed in CHO cells has been reported (60). However, in contrast to our results for EGFR phosphorylation, the transactivation of the PDGF receptor in CHO cells showed sensitivity to PTX (60). The D_3 receptor expressed in CHO cells mediates activation of PI 3-kinase via atypical protein kinase C in a manner also sensitive to PTX (63). In striatal neurons, the D_2 agonist has been reported to activate Akt independently of PI 3-kinase activation (65). However, we find in both PC12-D_2R and the dopaminergic SN4741 cells that Akt activation requires PI 3-kinase activity. Our results in the two dopamine cell lines studied are consistent with the observations of Kihara *et al.* (62) in cortical neurons, who also found that bromocriptine activated the PI 3-kinase/Akt pathway.

Our investigations further suggest that the D_2 receptor, when activated by bromocriptine, can couple both to heterotrimeric G_i/G_o family G-proteins and, simultaneously, to the PI 3-kinase/Akt signaling pathway (Fig. 11). These data led us to propose that the coupling to heterotrimeric G-protein and the coupling to PI 3-kinase/Akt may be independent. First, the G-protein coupling, but not the Akt activation showed PTX sensitivity. Second, the agonists studied differed in their capacity to activate each pathway. Bromocriptine activated both signaling pathways, whereas pramipexole, although quite efficient at simulating GTPγS binding in these cells (17), failed to activate the PI 3-kinase/Akt signaling pathway (see Fig. 10). Our data suggest that the anti-apoptotic activity induced by dopamine agonists in these cells resulted from transactivation of the PI 3-kinase/Akt pathway. In a previous study, we found little correlation between the capacity of agonists to confer protection against oxidative stress and their capacity to activate classic G-protein signaling (17). Based on these results, we

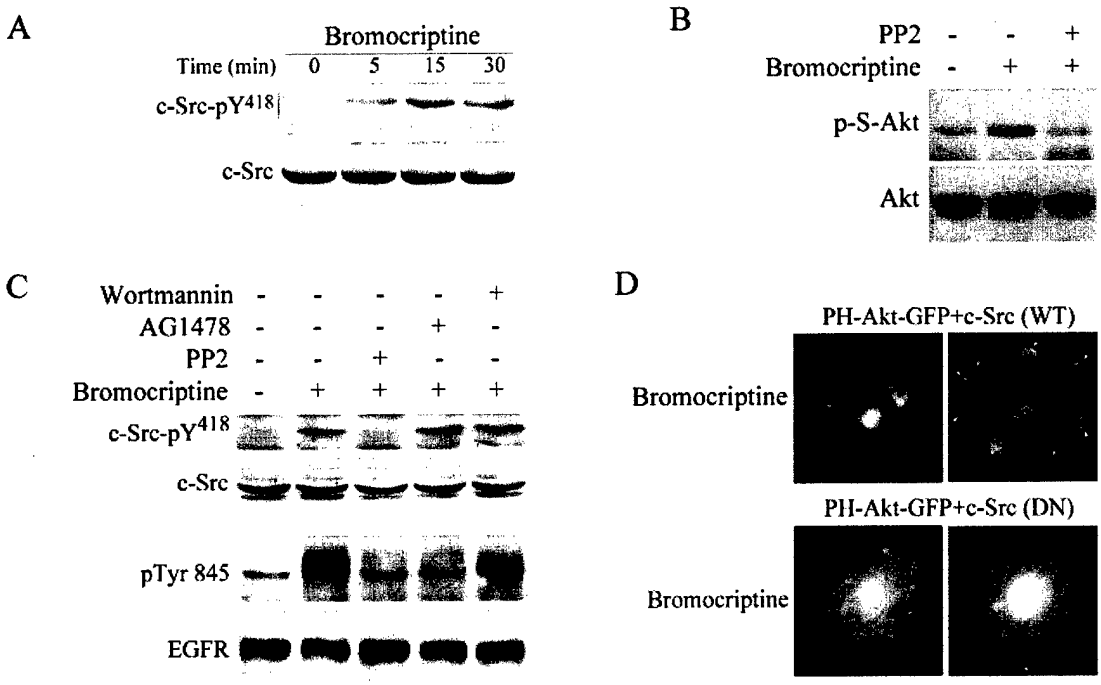

FIG. 9. **Bromocriptine stimulates the activation of the c-Src tyrosine kinase.** *A*, PC12-D_2R cells were incubated with 1 μM bromocriptine for the indicated time points. Tyrosine phosphorylation of site-specific tyrosine residues of c-Src was detected from total cell lysates using the anti-phospho Tyr^{418} antibody. Total c-Src level was detected using anti-Src antibody. *B*, PP2 treatment inhibits bromocriptine-induced phosphorylation of Akt. PC12-D_2R cells were either left untreated or pretreated with 1 μM PP2 for 30 min. The cells were then stimulated with 1 μM bromocriptine for 15 min. The cells were then lysed, and the lysates were resolved by SDS-gel electrophoresis and immunoblotted with either anti-phospho-Akt antibody or anti-Akt antibody. *C*, inhibition of Src-kinase abolished bromocriptine-mediated phosphorylation of c-Src and EGFR. *D*, inhibition of c-Src kinase activity prevents redistribution of PH-Akt-GFP in response to stimulation of the D_2 receptor by bromocriptine. PC12-D_2R cells were co-transfected with wild type Src or dominant negative Src (K295R/Y527F) and analyzed by epifluorescence microscopy. Redistribution of PH-Akt-GFP occurs in the presence of wild-type c-Src (*top panels*, see *arrows*) but not in the presence of dominant negative c-Src (*bottom panels*).

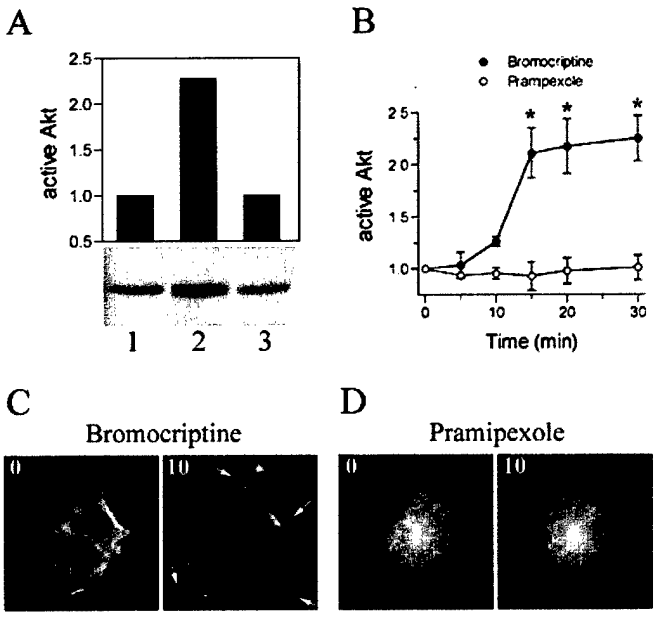

FIG. 10. **Differential phosphorylation of endogenous Akt by D_2 receptor agonists.** *A*, Western blot showing the differential phosphorylation of Akt by bromocriptine and pramipexole. *Lane 1*, control; *lane 2*, bromocriptine; and *lane 3*, pramipexole. The concentration of bromocriptine and pramipexole used was 1 μM for 30-min incubation. *B*, graphical representation of Akt phosphorylation in response to bromocriptine and pramipexole. Bromocriptine (1 μM) significantly increased (*, $p < 0.05$) Akt phosphorylation within 15 min after the addition of the drug, whereas pramipexole (1 μM) showed no effect on the phosphorylation of Akt. Data are mean ± S.E. values from one experiment performed in triplicate, representative of three independent experiments. *C* and *D*, in contrast with bromocriptine, pramipexole had no effect on the translocation of PH-Akt-GFP in PC12-D_2R cells.

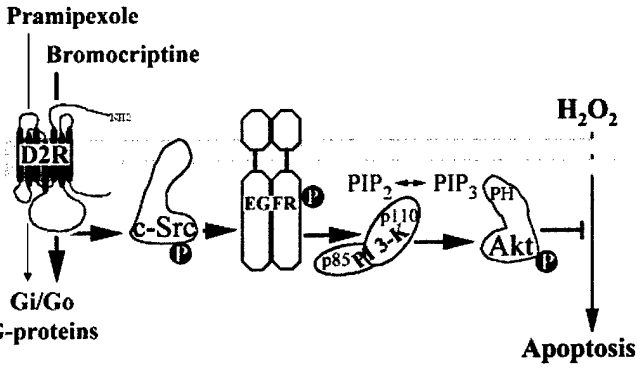

FIG. 11. **Schematic of signaling pathways modulated by the D_2 receptor.** Dopamine D_2 receptor activates PI 3-kinase/Akt pathway via c-Src and the EGFR. The D_2 agonist bromocriptine, but not pramipexole, activates the c-Src/EGFreceptor/PI 3-kinase/Akt pathway.

propose a model where the specificity of the agonist complexed with the D_2-receptor determines the switching of signaling between G-protein and growth factor signaling pathways.

Kenakin (13) originally proposed that specific agonists acting at the same receptor might differentially activate downstream signaling pathways, a phenomenon he called agonist-mediated signal trafficking. Signal trafficking could arise as a result of receptors having multiple active conformational states that differ in their activation of specific signaling pathways. Agonists could cause different patterns of signaling by each inducing a different relative distribution of the accessible active states. Many studies suggest that heptahelical receptors exhibit properties consistent with the existence of multiple conformational states. In rhodopsin, for example, the existence of multiple conformers is evident from absorbance changes (9).

Multiple receptor conformational states are also evident in single molecule spectroscopy studies of the β2-adrenergic receptor (11) and are supported by the presence of phenotypically different serotonin 5HT2C receptor activation mutants (12). Pharmacological evidence for signal trafficking has been reported in several heptahelical receptors (14, 15, 66, 67). Evidence for signal trafficking at the D_2 receptor based on the G-protein sensitivity of binding affinity has been previously reported for D_2 receptor expressing Sf21 insect cell lines. Notably, the agonist bromocriptine was found to induce a distinct pattern of coupling (15). Our results are consistent with the signal trafficking hypothesis and suggest that agonists acting at the D_2 receptor may differ markedly in their capacity to stabilize conformations leading to classic and growth factor signaling.

We implicate the capacity of D_2 agonists in transactivating the PI 3-kinase/Akt pathway and in mediating anti-apoptosis in PC12-D_2R cells. Furthermore, we find evidence that the effectiveness of an agonist to protect against oxidative stress by activating PI 3-kinase/Akt may differ greatly for specific agonists. Our results support the hypothesis that agonists have a conformationally specific effect at the D_2 receptor. Among the agonists studied to date, we find agonists that preferentially activate GTPγS binding and agonists that activate both GTPγS binding and anti-apoptotic signaling. Our results suggest that it may be possible to identify agonists that specifically traffic signaling to the EGFR-PI 3-kinase/Akt pathway in dopamine neurons. Given the central role of the dopamine D_2 receptor in brain function, the refined model of conformationally dependent D_2 receptor signaling has important implications for the pathophysiology and treatment of brain diseases involving altered dopamine neuronal survival or neurotransmission, such as Parkinson's disease and schizophrenia.

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